

## METHODS AND AGENTS FOR IMMUNE MODULATION AND METHODS FOR IDENTIFYING IMMUNE MODULATORS

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### FIELD OF THE INVENTION

The present invention relates to methods of regulating an immune response in an individual and, more particularly, to methods and agents which target NIK and NIK binding proteins participating in both the canonical and alternative NF- $\kappa$ B activation pathway, to methods of identifying molecules/agents for modulation of NIK activity and to the molecules/agents obtainable by the method thereof.

### BACKGROUND OF THE INVENTION

15 The NF- $\kappa$ B/Rel family of transcription factors is active in inflammatory and immune cell response, cell cycle regulation, differentiation and protection from apoptosis [Baeuerle and Baltimore, Cell 87:13–20, (1996); Ghosh, et al., Annu. Rev. Immunol. 16:225–260, (1998)]. In mammals, this family of transcription factors is comprised of five members: p65 (RelA), RelB, c-Rel, NF- $\kappa$ B1 (which occurs both as a precursor, p105, and in a processed form, p50) and NF- $\kappa$ B2 (which occurs both as a precursor, p100, and as its processed product, p52). The NF- $\kappa$ B protein homo- and heterodimers exist in the cytoplasm, in complex with inhibitors of the I $\kappa$ B family. The precursor forms of NF- $\kappa$ B1 and NF- $\kappa$ B2 (p105 and p100, respectively) contain C-terminal I $\kappa$ B-homologous inhibitory regions. Dimers containing these NF- $\kappa$ B proteins are retained in the cytoplasm by virtue of the function of the I $\kappa$ B-homologous regions. Moreover NF- $\kappa$ B1/p105 and NF- $\kappa$ B2/p100 can also associate with dimers of other NF- $\kappa$ B proteins and impose cytoplasmic retention on them. NF- $\kappa$ B activation occurs mainly through induced degradation of the I $\kappa$ B proteins or of I $\kappa$ B homologous regions in NF- $\kappa$ B1/p105 and NF- $\kappa$ B2/p100, and consequent translocation of the NF- $\kappa$ B dimers to the nucleus. The induced degradation of the I $\kappa$ B proteins provides the most important mechanism regulating NF- $\kappa$ B activity (Baeuerle and Baltimore, 1996) (Ghosh et al., 1998) (Ghosh and Karin, 2002).

Most of the knowledge of these processes concerns the mechanisms of activation of a ubiquitous NF- $\kappa$ B dimer, p65:p50. The critical event initiating this

'canonical' pathway is activation of an I $\kappa$ B-phosphorylating protein kinase, IKK2. IKK2 occurs within a macromolecular complex, the 'IKK signalosome', in association with a structurally homologous kinase, IKK1, and an adapter protein, NEMO. IKK2-mediated phosphorylation of I $\kappa$ B leads to its proteasomal degradation and hence  
5 activation of its associated NF- $\kappa$ B dimers (Karin and Ben-Neriah, 2000).

Other studies have yielded some knowledge of an 'alternative' pathway through which NF- $\kappa$ B dimers containing NF- $\kappa$ B2/p100 are activated. This activation occurs independently of IKK2 or NEMO, but is dependent on IKK1. Phosphorylation of p100 upon activation of this pathway leads to limited proteolytic processing in  
10 which only the I $\kappa$ B-homologous region within p100 is degraded. This process allows the resulting p52 fragment to translocate to the nucleus in association with some other NF- $\kappa$ B proteins (mainly RelB) (Xiao et al., 2001) (Senfleben et al., 2001) (Solan et al., 2002) (Coope et al., 2002) (Claudio et al., 2002) (Kayagaki et al., 2002) (Dejardin et al., 2002) (Yilmaz et al., 2003) (Hatada et al., 2003).

The proteins of the tumor necrosis factor/nerve growth factor (TNF/NGF) receptor family are a group of cell-surface receptors critically involved in the maintenance of homeostasis of the immune system. These proteins interact with their corresponding ligands, either to induce cell death or promote cell survival of immune cells. The biologic function of this group of proteins has been closely associated with  
20 the regulation of the immune response and the pathogenesis of autoimmune disease. [Zhou et al., Immunol. Res. 26:323-336, (2002)]. The TNF receptors control multiple immune-defense activities as well as certain developmental processes through NF- $\kappa$ B activation (Wallach et al., 1999) (Locksley et al., 2001). Most of these receptors are capable of activating the canonical NF- $\kappa$ B pathway. In addition, the lymphotoxin- $\beta$   
25 receptor (LT $\beta$ R), whose expression is restricted to stromal cells and several receptors that occur in lymphocytes (CD40, BLyS/BAFF and as shown in the present work - CD27), also activate the alternative pathway (Dejardin et al., 2002) (Coope et al., 2002) (Claudio et al., 2002) (Kayagaki et al., 2002) (Hatada et al., 2003).

Signaling for NF- $\kappa$ B activation by several receptors of the TNF receptor family  
30 is initiated by their binding to adapter proteins of the TRAF family. In cells treated with TNF the TRAFs have been shown to facilitate, collaboratively with the adapter protein RIP, recruitment of the signalosome components to the p55 TNF receptor

(Zhang et al., 2000) (Devin et al., 2000) (Devin et al., 2001). Additional protein that participates in NF- $\kappa$ B activation by the TNF/NGF receptor family was identified as a 'NF- $\kappa$ B-inducing kinase' (NIK), (Malinin et al., 1997).

Initially NIK was suggested to mediate activation of the canonical NF- $\kappa$ B pathway in response to multiple inducers with many different physiological functions (Malinin et al., 1997). However, later studies of mice of the *aly* strain, which express a non-functional NIK mutant, as well as of NIK-knockout mice, challenged the notion that NIK has a functional role in the activities of most of these inducers. They suggested rather, that NIK participates selectively in the activation of NF- $\kappa$ B by a restricted set of ligands that specifically affect the development and function of lymphocytes (Shinkura et al., 1999) (Yin et al., 2001). Moreover, based on characterization of cells derived from these mutant mice, it was suggested that NIK does not participate at all in the canonical NF- $\kappa$ B pathway, but rather serves exclusively to activate the alternative one (Pomerantz and Baltimore, 2002). Lymphocytes of NIK-mutant mice exhibit a highly aberrant pattern of differentiation (Miyawaki et al., 1994) (Shinkura et al., 1999) (Matsumoto et al., 1999) (Yamada et al., 2000) (Karrer et al., 2000) (Fagarasan et al., 2000), therefore, the present work aimed to re-assess the signaling role of NIK in lymphocytes.

In the present, the function of NIK in lymphocytes was now re-evaluated by assessing the effect of its depletion or inhibition *in vitro* in cultured cells of lymphoblastoid lines. The assays showing that NIK is not required for activation of the canonical pathway by TNF in lymphocytes were confirmed. However, as detailed below, NIK was found to play a crucial role in these cells in activation of the alternative as well as of the canonical pathway by CD40 ligand (CD40L) and BLyS/BAFF induction. Furthermore, CD27 (Camerini et al., 1991), a receptor of the TNF/NGF family that is expressed mainly in T lymphocytes and memory B lymphocytes and was previously suggested to activate NF- $\kappa$ B (Yamamoto et al., 1998) in a NIK-independent manner (Akiba et al., 1998) was shown to initiate the alternative pathway. It was also found by the inventors that NIK binds to SIVA, a protein associated with CD27 (Prasad et al., 1997), and mediates both the canonical and the alternative NF- $\kappa$ B-activating pathways in response to this receptor. Although NIK was not required for activation of the signalosome by the p55 TNF receptor, activation of

the signalosome by CD27 did depend on NIK. Moreover, unlike triggering by the p55 TNF receptor, triggering by CD27 induced, in a NIK-dependent way, selective recruitment of IKK1 to this receptor, a process that might be the initiating event in the NIK-dependent activation of both NF- $\kappa$ B pathways by CD27.

5       The biologic function of members of the NIK-dependent NF- $\kappa$ B pathway has been closely associated with the regulation of the immune response and the pathogenesis of autoimmune disease.

It is shown in accordance with the present invention that NIK, in contrast to prior art teachings, does participate in the canonical NF- $\kappa$ B activating pathway. In addition, it is shown that NIK participates in an alternative NF- $\kappa$ B pathway which is induced by BlyS and CD40L and have identified CD70 as a novel inducer of this alternative pathway.

As such, the present findings establish the role of NIK in NF- $\kappa$ B activation and thus provide the motivation to utilize various NIK targeting agents in treatment of various immune diseases.

#### SUMMARY OF THE INVENTION

The invention relates to the use of an agent capable of increasing or decreasing NIK-SIVA complex formation, in the manufacture of a medicament for the treatment of an immune disorder. More specifically, the said immune disorder is characterized by abnormal function or level of at least one protein selected from the group consisting of BlyS/BAFF, CD27, SIVA and NIK. Example of immune disorders according to the invention are multiple myeloma (MM), acquired immunodeficiency syndrome (AIDs), Sjogren's syndrome (SS), B-cells chronic lymphocytic leukemia (B-CLL), systemic lupus erythematosus, inflammatory colon disease, systemic inflammatory response syndrome (SIRS), multiple organ disinfection syndrome (MODS) and acute respiratory distress syndrome (ARDS).

In one aspect, the invention provides the use of an agent capable of increasing or decreasing NIK-dependent CD27 regulation in the manufacture of a medicament for treating an immune disorder. Particularly, the invention provides the use of an agent such as antibody capable of binding NIK, e.g. an antibody directed against the phosphorylated NIK activation loop, or a small interfering RNA molecule, e.g. that

set forth in SEQ ID NO: 15, or a ribozyme, for decreasing NIK-dependent CD27 regulation.

In another aspect, the invention provides the use of an agent capable of decreasing or increasing the activity of NIK in the manufacture of a medicament for treating an immune disorder caused or aggravated by the abnormal NF-kB activation via the canonical pathway. Particularly, the invention provides the use of an agent such as antibody capable of binding NIK, e.g. an antibody directed against the phosphorylated NIK activation loop, or a small interfering RNA molecule, e.g. that set forth in SEQ ID NO: 15, or a ribozyme, for decreasing NIK-dependent CD27 regulation.

More specifically, said abnormal NF-kB activation may be caused by induction of CD40L, Blys, CD70 and/or activation of the receptor thereof.

In addition, the invention provides a method of treating an immune disorder comprising administering to an individual having the immune disorder a therapeutically effective amount of an agent capable of increasing or decreasing NIK-SIVA complex formation, thereby treating the immune disorder in the individual. Particularly, said immune disorder is characterized by abnormal function or level of at least one protein selected from the group consisting of BlyS/BAFF, CD27, SIVA and NIK. More specifically, the method according the invention can be use to treat multiple myeloma (MM), acquired immunodeficiency syndrome (AIDs), Sjogren's syndrome (SS), B-cells chronic lymphocytic leukemia (B-CLL), systemic lupus erythematosus, inflammatory colon disease, systemic inflammatory response syndrome (SIRS), multiple organ disinfection syndrome (MODS) and acute respiratory distress syndrome (ARDS). In one embodiment of the invention, the administration of the agent modulating NIK-SIVA interaction can be effected by expressing said agent within cells, such as lymphocytes, of said individual.

Also, the invention relates to a method of treating an immune disorder comprising administering to an individual having the immune disorder a therapeutically effective amount of an agent capable of increasing or decreasing NIK-dependent CD27 regulation, thereby treating the immune disorder in the individual.

In particular, the administration may be effected by expressing said agent within cells such as lymphocytes of said individual.

In a further embodiment, the invention relates to a method of treating an immune disorder caused or aggravated by abnormal NF-kB activation by the canonical pathway, comprising administering to an individual suffering from the disorder a therapeutically effective amount of an agent capable of decreasing or increasing the activity of NIK. Particularly, the abnormal NF-kB activation may be caused by induction of CD40L, CD70, or Blys and/or activation of the receptor thereof.

In one particular embodiment of the invention, the method involves the use of an agent capable of decreasing the activity of NIK, for example an antibody directed against the phosphorylated NIK activation loop, a small interfering RNA molecule such as the one of SEQ ID NO: 15, or a ribozyme.

The invention provides also, an isolated polynucleotide comprising a nucleic acid sequence capable of specifically down-regulating NIK expression in cells provided thereto such as a small interfering RNA molecule like the one of SEQ ID NO:15, a construct comprising such polynucleotide and a cell comprising the nucleic acid construct.

In another embodiment, the invention provides an antibody or antibody fragment capable of specifically binding to an amino acid sequence region set forth by coordinates 624-947 of SEQ ID NO:2, 123-175 of SEQ ID NO:3 and/or 58-110 of SEQ ID NO:4

In addition, the invention provides a method of identifying a putative immune modulator, the method comprising identifying a molecule capable of increasing or decreasing NIK-SIVA complex formation, said molecule being the putative immune modulator.

Also the invention provides a method of identifying a putative immune modulator, the method comprising identifying a molecule capable of increasing or decreasing NIK-dependent CD27 regulation, said molecule being the putative immune modulator.

Moreover the invention provides a method for the screening (or identification and/or selection ) of molecules capable of modulating the activity of NIK comprising contacting a cell with a ligand of a TNF/NGF receptor family capable to induce NIK-dependent canonical and alternative pathway in the cell, incubating the cell prior to, after, or during said contacting with individual tested molecules, detecting activation of the canonical pathway in the cell and selecting individual molecule/s capable of modulating induction of the canonical pathway induced by said ligand.

- 10 In one aspect, the invention provides a method for the screening (identification and/or selection) of molecules capable of modulating NIK activity comprising contacting a lymphoblastoid cell with a ligand of a TNF/NGF receptor family capable of activating NIK and the canonical pathway in the cell, incubating the cell prior to, after, or during said contacting, with individual tested molecules, detecting activation of the canonical pathway and, selecting individual molecule/s capable of modulating induction of the canonical pathway induced by said ligand but not by any other ligand capable of inducing canonical pathway in a NIK independent manner.

In one embodiment of the invention, the ligand used for the screening method is selected from CD70, CD40L, or Blys/BAFF.

In another embodiment of the invention, the cells for the screening method are of a lymphoblastoid type such as for e.g. Ramos, Raji or BJAB cells.

- 25 In a further embodiment of the invention, activation of the canonical pathway is detected in the screening method by monitoring parameters indicative of the canonical pathway activation, such as I $\kappa$ B degradation, I $\kappa$ B $\alpha$  phosphorylation and p65 translocation.

30 Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable

methods and materials are described below. In case of conflict, the patent specification, including definitions, will control. In addition, the materials, methods, and examples are illustrative only and not intended to be limiting.

## 5 BRIEF DESCRIPTION OF THE DRAWINGS

The invention is herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of the preferred embodiments of the present invention only, and  
10 are presented in the cause of providing what is believed to be the most useful and readily understood description of the principles and conceptual aspects of the invention. In this regard, no attempt is made to show structural details of the invention in more detail than is necessary for a fundamental understanding of the invention, the description taken with the drawings making apparent to those skilled in  
15 the art how the several forms of the invention may be embodied in practice.

In the drawings:

FIGs. 1a-e illustrate binding of NIK to SIVA.

Figure 1a illustrates yeast two-hybrid binding assays of NIK to SIVA. The binding of NIK and its C-terminally mutant (NIK 624–947) to the C-terminal part of SIVA  
20 (amino acids 123–175 in SIVA1 or 58–110 in SIVA2) or TRAF2, was assessed using transformed SFY526 yeast. The development of a strong color reaction within 1 hour and 3 hours is indicted as ‘++’ and ‘+’, respectively; ‘–’ indicates no color development within 24 h. This assay shows that the C-terminal SIVA fragment binds to the C-terminal part of NIK and this binding is stronger than that observed with the  
25 full-length NIK protein.

The top panel of Figure 1b is a table representing the transfection pattern of HEK293T cells with plasmids expressing myc-NIK, HIS-SIVA1, HIS-SIVA2 or myc-*aly* NIK. ‘+’ indicates that the corresponding plasmid was used for transfection, otherwise ‘–’ is indicated.

30 The middle panel of Figure 1b represents a co-immunoprecipitation of NIK (or of NIK into which a missense mutation corresponding to that found in *aly* mice was introduced) with SIVA using antibodies against the HIS fused to SIVA1 and SIVA2. Co-immunoprecipitation was assessed after 24h.



The bottom panel of Figure 1b is a western blot analysis on total cell lysate with antibodies against myc tag fused to NIK and *aly* NIK.

The top panel of Figure 1c is a table representing the transfection pattern of HEK293T cells with plasmids expressing HIS-SIVA1, HIS-SIVA2 myc-NIK, or myc-*aly* NIK.

5 '+' indicates that the corresponding plasmid was used for transfection, otherwise '-' is indicated.

The middle panel of Figure 1c is a co-immunoprecipitation of SIVA with NIK from transiently transfected HEK293T cells using antibodies against the myc fused to NIK and *aly* NIK, co-immunoprecipitation was assessed after 24h. The bottom panel of

10 Figure 1c is a western blot analysis on total cell lysate with antibodies against HIS tag fused to SIVA1 and SIVA2. Figures 1b and 1c show that NIK co-immunoprecipitate bidirectionally with SIVA1 and SIVA2 and '*aly* NIK' co-immunoprecipitate with SIVA1 and to a small extent also with SIVA2.

The top panel of Figure 1d is a table representing the transfection pattern of  
15 HEK293T cells with plasmids expressing myc-NIK, HIS-hIKK1, pEGFP, pcHIS-SIVA1 or pcHIS-SIVA2. '+' indicates that the corresponding plasmid was used for transfection, otherwise '-' is indicated.

The bottom panel of Figure 1d is a western blot analysis on total cell lysate using antibodies against the myc tag fused to NIK. This figure demonstrates that the  
20 quantity of NIK in the transfected cells is increased by the co-expression with SIVA1 or SIVA2. Figure 1e is a bar graph illustrating the enhancement of NIK-mediated NF- $\kappa$ B activation by co-expressed SIVA. The effect of over-expression of NIK or *aly* NIK, alone or together with SIVA1 or SIVA2, on HIV-luciferase expression in HEK293T cells was assessed 24 hours after transfection. Values are the means  
25 obtained in two experiments in which each test was carried out in triplicate. The graph shows that SIVA is capable of affecting NIK function.

FIGs. 2a-h illustrate the induction of both the canonical and the alternative pathways in lymphocytes by CD70 (CD27 ligand) and the effect of NIK deficiency on this induction.

30 Figure 2a is a western blot analysis designed for detecting I $\kappa$ B $\alpha$  levels in the cytoplasm of resting PBMC following CD70 application and p52 and RelB levels in the nucleus of resting PBMC following CD70 treatment. This figure demonstrates

rapid decrease of I $\kappa$ B $\alpha$  as well as translocation of NF- $\kappa$ B2/p52 (p52) and RelB to the nuclei.

Figure 2b is a western blot analysis designed for detecting I $\kappa$ B $\alpha$  levels in the cytoplasm of stimulated PBMC and p100, p52 and RelB levels in the nucleus of stimulated PBMC upon CD70 treatment. This figure shows rapid decrease of I $\kappa$ B $\alpha$ .

Figure 2c is a western blot analysis designed for detecting I $\kappa$ B $\alpha$  levels in the cytoplasm of Raji cells and p100, p52, RelB and p65 levels in the nucleus of Raji cells upon CD70 treatment. This figure shows that I $\kappa$ B $\alpha$  degradation as well as nuclear translocation of RelB and NF- $\kappa$ B2/p52.

Figure 2d is a western blot analysis designed for detecting p100 and p52 levels in the cytoplasm of normal and NIK<sup>(MINUS)</sup> Ramos cells and p100, p52 and RelB levels in the nucleus of these cells upon CD70 treatment. This figure demonstrates the induction of nuclear translocation of RelB and NF- $\kappa$ B2/p52 in normal Ramos cells, as well as delayed p100 nuclear translocation in NIK<sup>(MINUS)</sup> Ramos cells. Figure 2e is a western blot analysis designed for detecting I $\kappa$ B $\alpha$  levels in the cytoplasm of normal and NIK<sup>(MINUS)</sup> Ramos cells and p65 levels in the nucleus of these cells upon CD70 treatment. This figure demonstrates I $\kappa$ B $\alpha$  degradation as well as nuclear translocation of p65 in normal Ramos cells.

Figure 2f demonstrates the suppression of NIK synthesis by expression of NIK siRNA.

The top panel of Figure 2f depicts a western blot analysis designed for detecting NIK levels in HEK293 cells transiently expressing myc-tagged NIK and co-transfected with pSUPER-NIK at ratios of 1:1, 1:2, 1:3 and 1:5. This figure shows that NIK is effectively suppressed.

The middle panel of Figure 2f depicts a western blot analysis designed for detecting NIK levels in Ramos cells constitutively expressing lentiviral-pSUPER-NIK (NIK<sup>(MINUS)</sup> cells) in comparison to Ramos cells transduced with lentiviral-GFP as control. This figure shows that NIK is effectively suppressed.

The bottom panel of Figure 2f depicts a western blot analysis designed for detecting NIK levels in NIK<sup>(MINUS)</sup> Ramos cells to which NIK expression was reinstated by constitutively expressing myc-tagged NIK. This figure demonstrates that NIK expression was reinstated.

Figure 2g is a bar graph demonstrating CD70-induced protein kinase C (PKC) activation in normal (black bars) and NIK<sup>(MINUS)</sup> (white bars) Ramos cells. PKC activation was performed in cell lysate using the Signatect PKC assay system at various time points (0, 15 and 30 minutes) following CD70 application to the cells. Bars represent the means of triplicate tests. CD27 levels in normal and NIK<sup>(MINUS)</sup> Ramos cells are shown in the inset. This figure shows that NIK<sup>(MINUS)</sup> Ramos cells express CD27 at levels comparable to those in normal Ramos cells and manifest a normal extent of protein kinase C (PKC) activation upon CD27 triggering. Figure 2h is a western blot analysis designed for detecting IκBα levels in the cytoplasm of NIK<sup>(MINUS)</sup> reconstituted Ramos cells and p52 levels in the nucleus of these cells. This figure demonstrates these cells regain the ability to respond to CD70 with both an increase in nuclear p52 and a transient decrease in IκBα.

FIGs. 3a-i demonstrate the induction of both the canonical and the alternative NF-κB pathways by CD40L, BLyS/BAFF, TNF, thapsigargin or PMA and the effect of NIK deficiency on this induction.

Figure 3a is a western blot analysis designed for detecting p100, p52 and RelB levels in the nucleus of normal and NIK<sup>(MINUS)</sup> Ramos cells following CD40L treatment. This figure demonstrates induction of nuclear translocation of p100, p52 and RelB in normal Ramos cells.

Figure 3b is a western blot analysis designed for detecting IκBα levels in the cytoplasm of normal and NIK<sup>(MINUS)</sup> Ramos cells and p65 levels in the nucleus of these cells following CD40L treatment. This figure demonstrates rapid induction of nuclear translocation of p65 associated with a decrease in IκBα in normal Ramos cells.

Figure 3c is a western blot analysis designed for detecting p100, p52 and RelB levels in the nucleus of normal and NIK<sup>(MINUS)</sup> Ramos cells following BLyS treatment. This figure demonstrates the induction of nuclear translocation of p52 and RelB in normal Ramos cells.

Figure 3d is a western blot analysis designed for detecting phosphorylated IκBα levels in the cytoplasm of normal and NIK<sup>(MINUS)</sup> Ramos cells and p65 levels in the nucleus of these cells following BLyS treatment. The figure demonstrates rapid

nuclear translocation of p65 associated with phosphorylation of I $\kappa$ B $\alpha$  with no visible change in its cellular levels in normal Ramos cells.

Figure 3e is a western blot analysis designed for detecting p100, NS, p52 and RelB levels in the nucleus of normal and NIK<sup>(MINUS)</sup> Ramos cells following TNF treatment.

5 This figure demonstrates induction of nuclear translocation of p100 and RelB but only a slight increase in nuclear p52 in normal Ramos cells and a nuclear translocation of p100 and RelB in NIK<sup>(MINUS)</sup> Ramos cells.

Figure 3f is a western blot analysis designed for detecting I $\kappa$ B $\alpha$  levels in the cytoplasm of normal and NIK<sup>(MINUS)</sup> Ramos cells and p65 levels in the nucleus of  
10 these cells following TNF treatment. The figure shows the induction of I $\kappa$ B $\alpha$  degradation and nuclear translocation of p65 in both, normal and NIK<sup>(MINUS)</sup> Ramos.

Figure 3g is an immunoprecipitation analysis with RelB of various NF- $\kappa$ B proteins from nuclear extracts of the Ramos cells, 15 minutes and 4 hours after the application of TNF or CD70 to the cells. Levels of p100, NS and p52 were detected by western  
15 blot analysis. This figure demonstrates that CD70 enhances nuclear accumulation of RelB:p52 and RelB:p100 while TNF induces increased nuclear levels of only RelB:p100.

Figure 3h is a western blot analysis designed for detecting I $\kappa$ B $\alpha$  levels in the cytoplasm of normal and NIK<sup>(MINUS)</sup> Ramos cells in response to thapsigargin or 4 $\beta$ -  
20 phorbol-12-myristate-13-acetate (PMA). This figure demonstrates that NIK depletion has no effect on I $\kappa$ B $\alpha$  degradation.

Figure 3i is a western blot analysis designed for detecting basal levels of p100, p52, p65, RelB, c-Rel and I $\kappa$ B $\alpha$  in normal and NIK<sup>(MINUS)</sup> Ramos cells. This figure demonstrates marked reduction of basal p52 and a significant decrease in RelB and c-  
25 RelB as well as some reduction in p100 and I $\kappa$ B $\alpha$  in NIK<sup>(MINUS)</sup> Ramos cells as compared with normal Ramos cells.

FIGs. 4a-d demonstrate that the induction of I $\kappa$ B $\alpha$  degradation by CD40L and BLyS and not by TNF is blocked by  $\alpha$ -pNIK antibodies against the phosphorylated activation loop.

30 Figure 4a is an autoradiogram of the phosphorylated protein as compared with a western blot analysis of NIK levels in the same samples. Self-phosphorylation of myc-NIK immunoprecipitated from transiently transfected HEK293T cells in the

presence of 0  $\mu$ g, 0.5  $\mu$ g, 1.0  $\mu$ g and 2  $\mu$ g of  $\alpha$ -pNIK antibodies or with control 2  $\mu$ g IgG. This figure demonstrates that  $\alpha$ -pNIK effectively blocks the *in-vitro* kinase function of NIK. Figure 4b demonstrates introduction of antibodies into Ramos cells using a protein-transfection reagent.

5 Figure 4b is a photograph of uptake of FITC-tagged immunoglobulin by Ramos cells, assessed by fluorescent microscopy at various times (0, 1, 4 and 8 hours) after transfection. This figure demonstrates that treatment of Ramos cells with a protein-transfection kit allows effective, though transient, introduction of immunoglobulins into the cells.

10 Figure 4c is a western blot analysis designed for detecting degradation of I $\kappa$ B $\alpha$  induced by CD70, CD40L or TNF in Ramos cells with  $\alpha$ -pNIK antibody. This figure demonstrates that  $\alpha$ -pNIK antibody effectively blocks the induction of I $\kappa$ B $\alpha$  degradation by CD70 or CD40L.

Figure 4d is a western blot analysis designed for detecting degradation of I $\kappa$ B $\alpha$  by  
15 CD40L in BJAB cells with  $\alpha$ -pNIK antibody. This figure demonstrates that CD40L induces I $\kappa$ B $\alpha$  degradation in these cells and this induction is significantly reduced in the presence of  $\alpha$ -pNIK antibodies.

FIGs. 5a-d demonstrate the effect of CD70 or TNF on recruitment of IKK's and activation of the IKK signalosome in normal and NIK<sup>(MINUS)</sup> Ramos cells.

20 Figure 5a (top panel) is a kinetic analysis of *in-vitro* I $\kappa$ B $\alpha$  phosphorylation activity of the IKK signalosome in Ramos cells, isolated by immunoprecipitation using antibodies to IKK1, as compared with western blot analysis (bottom panel) designed for detecting cellular I $\kappa$ B $\alpha$  levels, at indicated times (0, 15 minutes or 4 hours) after application of CD70 or TNF to normal and NIK<sup>(MINUS)</sup> Ramos cells. This figure  
25 demonstrates that both TNF and CD70 enhance the *in vitro* kinase function of the IKK signalosome in normal Ramos cells. In NIK deficient Ramos cells, CD70 induced activation of the signalosome was blocked and there was no *in-vitro* I $\kappa$ B phosphorylation, while TNF-induced activation of the signalosome was not affected at all.

30 Figure 5b demonstrates self-phosphorylation of the IKKs and phosphorylation of NEMO in *in-vitro* kinase test of the IKK signalosome isolated 15 minutes after application of CD70 or TNF to normal and NIK<sup>(MINUS)</sup> Ramos cells. This figure

demonstrates that both TNF and CD70 enhance self-phosphorylation of the IKKs and phosphorylation of NEMO in normal Ramos cells. The effect of CD70 on the signalosome is aborted in the NIK<sup>(MINUS)</sup> Ramos cells.

Figure 5c demonstrates the recruitment of IKK1, IKK2 and NEMO by CD70 or TNF induction in normal and NIK<sup>(MINUS)</sup> Ramos cells. The top panel of Figure 5c demonstrates *in-vitro* IκBα phosphorylation activity and presence of the IKK signalosome components in the receptor complexes associated with CD27 (left) and the p55 TNF receptor (right) isolated from normal and NIK<sup>(MINUS)</sup> Ramos cells before and after stimulation with CD70 or TNF for 15 min.

The bottom panel of Figure 5c demonstrates *in-vitro* IκBα phosphorylation activity and western blot analysis designed for detecting the IKK signalosomes isolated from Ramos cells at the same times as the receptor complexes were isolated. The amounts of IKK1 introduced into the kinase tests corresponded to those shown in this figure. This figure demonstrates that TNF induces the recruitment of all three components of the signalosome (IKK1, IKK2, and NEMO), in about the same ratio as that found in the complex that they form in the cytosol both in normal and NIK<sup>(MINUS)</sup> Ramos cells. CD70 induces the recruitment of only IKK1 in normal Ramos cells.

Figure 5d demonstrates an *in-vitro* IκBα phosphorylation activity and a western blot analysis designed for detecting the presence of IKK signalosome components in the receptor complexes associated with CD27 and signalosome preparations isolated from resting PBMC before and after stimulation with CD70 for 15 min. This figure demonstrates that CD70 induces the selective recruitment of IKK1.

FIGs. 6 a-d demonstrate that CD70 induces recruitment of the IKK signalosome followed by selective recruitment of IKK1 to CD27 in a way that depends on NIK kinase function, as well as recruitment of NIK independently of its kinase function.

Figure 6a shows a kinetic analysis of recruitment of TRAF2 and RIP, the components of the IKK signalosome (IKK1, IKK2, and NEMO), the components of the canonical NF-κB complex (IκBα, p65, and p50), and p100 to CD27 and p55 TNF receptor complexes in Ramos cells at various time points after CD70 or TNF application, compared to composition of the cytoplasmic IKK signalosome (isolated, prior to

stimulation, by the use of antibody to NEMO; right), and to the cellular levels of I $\kappa$ B $\alpha$  (bottom).

Figures 6b, 6c show in-vitro I $\kappa$ B phosphorylation activity and the presence of IKK signalosome components in the receptor complexes and cytoplasmic signalosomes.

5 Figure 6b shows CD27 complexes and signalosome preparations isolated from resting PBMC before stimulation, and after stimulation with CD27 for 20 min.

Figure 6c shows the receptor complexes associated with CD27 (left) and p55 TNF receptor (right) isolated from control and NIK- Ramos cells before stimulation, and after stimulation with CD70 or TNF for 20 min.

10 Figure 6d shows the comparison of the kinetics of recruitment of NIK and IKK1 to CD27 and to the p55 TNF receptor complexes at various times after application of CD70 or TNF to NIK- cells replenished with wild type or enzymatically inactive NIK mutant (KD-NIK).

15 FIG. 7 depicts a speculative model of the mechanisms initiating NF- $\kappa$ B activation by TNF (left panel) and CD70 (right panel). The figure presents an outline of the molecular events leading from activation of the p55 TNF receptor by TNF (left) and of the CD27 receptor by CD70 (right) to NF- $\kappa$ B activation. TNF induces NIK-independent recruitment of all three core components of the signalosome to its  
20 receptor in a way that depends on interaction of these components with TRAFs and RIP. This recruitment initiates the canonical pathway only. CD70 induces recruitment and massive ubiquitination of TRAF2, but not RIP. It also induces the recruitment of NIK and, in a way that depends on the kinase function of NIK, induces also the recruitment first of the whole signalosome and then of only IKK to CD27.  
25 Recruitment of whole signalosome to this receptor and the consequent activation of IKK1 in it by NIK might be the mechanism for initiation by this receptor of the canonical pathway, and the subsequent recruitment of IKK1 might be the mechanism for initiation by this receptor of the alternative pathway. Broken lines represent the induction of p100 and RelB upon activation of the canonical pathway by TNF and  
30 CD70 and the consequent translocation of the p100:RelB complex to the nucleus.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The present invention relates to the use of agents capable of increasing or decreasing the activity of NIK in immune disorders caused or aggravated by abnormal NF- $\kappa$ B activation via the canonical pathway. In another aspect, the invention relates to the use of an agent capable of increasing or decreasing NIK-SIVA complex formation in the treatment of immune disorders.

The present invention relates also to methods for the screening (identification and/or selection) of molecules capable of modulating (increasing or decreasing) the activity of NIK, and to the molecules obtainable by the methods thereof.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.

The NF- $\kappa$ B family of transcription factors are associated with a large number of biological functions including inflammatory and immune cell response, cell cycle regulation, differentiation and protection from apoptosis [Baeuerle and Baltimore, Cell 87:13–20, (1996); Ghosh, et al., Annu. Rev. Immunol. 16:225–260, (1998)]. The majority of these activities have been realized from studies of NF- $\kappa$ B function in regulation of lymphocyte survival and activation.

It is well established that controlled activation of NF- $\kappa$ B is essential for normal innate and adaptive immune responses, and that abnormal regulation of NF- $\kappa$ B signaling in lymphocytes results in development of diseases ranging from chronic inflammation and autoimmunity to lymphoma [Ruland, and Mak, Semin. Immunol. 3:177–83, (2003)]. Accordingly, arrest of NF- $\kappa$ B signals by blocking ligand–receptor interactions enables effective suppression of signaling activities which are associated with T and B lymphocyte activation and growth, inflammation, fibroblast proliferation, and cell death. Therefore, regulation of NF- $\kappa$ B activities can be proven



beneficial to treatment of various disorders, which are associated with the above described cell signaling activities.

NF- $\kappa$ B activation results from the activation of at least one of two parallel signaling pathways, termed canonical and alternative, described in details in the preceding Background section.

One of the key elements in NF- $\kappa$ B activation is the NF- $\kappa$ B inducing kinase (NIK). While this protein has been initially implicated in the activation of the canonical NF- $\kappa$ B pathway in response to multiple inducers [N. L. Malinin, M. P. Boldin, A. V. Kovalenko, D. Wallach, *Nature* **385**, 540-4 (1997); H. Akiba *et al.*, *J Biol Chem* **273**, 13353-8. (1998)], these referenced prior art studies were based on the ability of overexpressed NIK mutants to block signaling, an approach which is now considered unreliable as is evident from the fact that the same experimental approach provided evidence that NIK functions in TNF activation of the canonical pathway, a finding that has since then been refuted [L. Yin *et al.*, *Science* **291**, 2162-5. (2001)].

Thus, more recent studies refute early findings and provide overwhelming evidence that NIK does not participate in activation of the canonical pathway and that studies that suggested that NIK participates in CD27 signaling were erroneous [S. Ghosh, M. Karin, *Cell* **109** Suppl, S81-96 (Apr, 2002); E. Dejardin *et al.*, *Immunity* **17**, 525-35 (Oct, 2002) and J. L. Pomerantz, D. Baltimore, *Mol Cell* **10**, 693-5 (Oct, 2002).

Thus, although NIK inhibition has been suggested as a possible therapeutic approach in treatment of systemic inflammatory response syndrome, it is highly unlikely that NIK inhibitory agents will be utilized as efficacious drugs, in diseases which are caused or aggravated by NF- $\kappa$ B activation through the canonical pathway, since at present the scientific community clearly doubts the role of NIK in activating the canonical pathway.

In accordance with the present invention it has been established that NIK, in contrast to prior art teachings, does participate in the canonical NF- $\kappa$ B activating pathway. In addition, it has been found that NIK also participates in activation of the alternative NF- $\kappa$ B pathway via CD70/CD27 signaling.

The present findings establish a role for NIK in NF- $\kappa$ B activation and thus provide the motivation to utilize various NIK targeting agents in treatment of various immune diseases, which are caused or aggravated by NF- $\kappa$ B activation.

As is illustrated in the Examples section which follows, the present inventors have established that NIK plays a crucial role in activation of the alternative as well as of the canonical pathway by CD40 ligand (CD40L), BLyS/BAFF and CD27. Furthermore, NIK was found to bind SIVA, a protein associated with CD27 (Prasad et al., 1997), and to thereby mediate both the canonical and the alternative NF- $\kappa$ B-activating pathways in response to this receptor. Although NIK was not required for activation of the signalosome by the p55 TNF receptor, activation of the signalosome by CD27 did depend on NIK. Moreover, unlike triggering by the p55 TNF receptor, triggering by CD27 induced, in a NIK-dependent way, selective recruitment of IKK1 to this receptor, a process that might be the initiating event in the NIK-dependent activation of both NF- $\kappa$ B pathways by CD27.

Elucidation of the alternative and canonical NF- $\kappa$ B activating pathways which is enabled by the present study (see Figure 6), allows for the design of refined therapies aimed to specifically blocking the deleterious effects of unregulated activity of the transducers and effectors of these pathways.

Thus, the present invention provides a method of treating an immune disorder in an individual.

As used herein the phrase "immune disorder" refers to a disorder associated with insufficient or excessive antigen-specific or antigen non-specific (i.e., innate) immune response in which there is an abnormal activity of at least one protein (further described hereinbelow) participating in a NIK-dependent NF- $\kappa$ B signaling (i.e., canonical and alternative pathways, such as illustrated in Figure 6). Examples of such disorders include, but are not limited to, multiple myeloma (MM), acquired immunodeficiency syndrome (AIDs), Sjogren's syndrome (SS), B-cells chronic lymphocytic leukemia (B-CLL), systemic lupus erythematosus, inflammatory colon disease, systemic inflammatory response syndrome (SIRS), multiple organ disinfection syndrome (MODS) and acute respiratory distress syndrome (ARDS), Addison's disease, allergies, ankylosing spondylitis, amyloidosis, anemia, asthma, atherosclerosis, autoimmune hemolytic anemia, autoimmune thyroiditis, bronchitis, cholecystitis, contact dermatitis, Crohn's disease, atopic dermatitis, dermatomyositis,

diabetes mellitus, emphysema, erythema nodosum, atrophic gastritis, glomerulonephritis, Goodpasture's syndrome, gout, Graves' disease, Hashimoto's thyroiditis, hypereosinophilia, irritable bowel syndrome, multiple sclerosis, myasthenia gravis, myocardial or pericardial inflammation, osteoarthritis, osteoporosis, pancreatitis, polymyositis, rheumatoid arthritis, scleroderma, systemic anaphylaxis, systemic sclerosis, ulcerative colitis, Werner syndrome, and complications of cancer, hemodialysis, and extracorporeal circulation; viral, bacterial, fungal, parasitic, protozoal, and helminthic infections; and trauma.

As used herein the term "treating" refers to preventing, curing, reversing, attenuating, alleviating, minimizing, suppressing or halting the deleterious effects of an above-described immune disorder.

As used herein the term "individual" refers to a mammal, preferably a human.

According to the present invention, an individual can be provided with a therapeutically effective amount of an agent capable modulating the activity of a target gene or a target gene product (i.e., RNA or protein) participating in a NIK-dependent NF- $\kappa$ B signaling, thereby treating the immune disorder in the individual.

As used herein the phrase "modulating the activity" refers to increasing or decreasing an intrinsic catalytic activity (e.g., kinase activity of NIK), interacting activity (e.g., NIK-SIVA interaction as illustrated in Example 1 of the Examples section) or expression (e.g., NIK expression as illustrated in Example 2 of the Examples section) of the target gene or target gene product.

A number of genes and their products can be used as targets in accordance with the present invention (see Figure 6). Examples of such target genes are listed below along with examples immune disorders involving same.

**BLyS** – BLyS binds the BAFF-receptor protein and promotes the survival of mature B-cells and B-cell Response. The protein is abundantly expressed in peripheral blood leukocytes and is specifically expressed in monocytes and macrophages. It is also found in the spleen, lymph node, bone marrow, T-cells and dendritic cells. The involvement of B lymphocyte stimulator (BLyS) in multiple myeloma (MM) was demonstrated in several aspects. MM cells were shown to express BLyS receptors and BLyS, in turn, was shown to modulate proliferative capacity and survival of MM cells. BLyS protein was also found in the bone marrow of MM patients [Novak et al., Blood. Epub ahead of print (2003)]. BLyS levels

together with globulin were also found to increase as HIV disease progresses [Rodriguez et al., AIDS. 17:1983-1985 (2003)]. The involvement of BLyS molecule in another autoimmune disease, Sjogren's syndrome (SS), was demonstrated by its ability to mediate polyclonal activation of B lymphocytes, and its role in the production of auto-antibodies. It was also shown that in human SS patients, the level of BLyS correlates with the level of auto-antibodies. Thus, BLyS may play a part in activating specific auto-reactive B cells and modulating the level of production of auto-antibodies which are the hallmark of the disease [Mariette et al., Ann. Rheum. Dis. 62:168-171, (2003)]. Another disease, in which BLyS was shown to play a role, is systemic lupus erythematosus. Over-expression of BLyS in mice leads to a systemic-lupus-erythematosus-like (SLE-like) disease. Over-expression of BLyS is also common in human SLE. Treatment of SLE-prone mice with a BLyS antagonist ameliorates disease progression and enhances survival [Stohl, Arthritis Res. Ther. 5:136-138, (2003)]. An effect of BLyS was demonstrated in B-cell chronic lymphocytic leukemia (B-CLL), a disease characterized by accumulation of CD5(+) B cells in the periphery and bone marrow. All B-CLL patient cells studied, expressed one or more of 3 known receptors for BLyS. B-CLL cells from a subset of patients aberrantly express BLyS and APRIL mRNA, whereas these molecules were not detectable in normal B cells. In addition, BLyS was found to protect B-CLL cells from apoptosis and to enhance cell survival [Novak et al., Blood. 100:2973-2979, (2002)]. Heterotrimeric of two proteins, APRIL and BLyS were found in serum samples from patients with systemic immune-based rheumatic diseases, implicating a role for these molecules also in rheumatic diseases [Roschke et al., J. Immunol. 169:4314-4321, (2002)]. Thus, the present invention envisages down-regulation of BLyS signaling through NIK-dependent NF- $\kappa$ B pathway to overcome the above-described immune disorders.

**CD40L** – This ligand can activate NIK-dependent NF- $\kappa$ B signaling (see Example 6 of the Examples section) through binding to the CD40 receptor. CD40L was shown to be involved in HIV infection. It was suggested that reversing the relative CD40L deficiency seen in HIV infection can facilitate immune restoration in AIDS [Kornbluth, J. Leukoc. Biol. 68:373-382, (2000)]. Thus, the present invention envisages up-regulation of CD40L signaling through NIK-dependent NF- $\kappa$ B pathway to overcome the above-described immune disorders.

*CD27* – Expression of CD27, by B-cell chronic lymphocytic leukemia (B-CLL) cells have been shown to influence clinical outcome of this disease [Bannerji and Byrd, Curr. Opin. Oncol. 12:22-29, (2000)]. CD27 was also shown to have heterogeneous expression in multiple myeloma patients. Low CD27 expression was found to correlate with patients with high-risk disease [Guikema et al., Br. J. Haematol. 121:36-43, (2003)]. CD27 was also found in systemic lupus erythematosus patients in relation with lymphocytes count and disease course [Swaak et al., Clin. Rheumatol. 14:293-300, (1995)]. Thus, the present invention envisages selected up-regulation or down-regulation of CD27 signaling through NIK-dependent NF- $\kappa$ B pathway according to the immune disorder to be treated.

*NIK* – “NF- $\kappa$ B inducing kinase” binds SIVA, TRAF2, TRAF5, TRAF6, IKKA AND NF-kappa-B<sup>2</sup>/P100. This protein is weakly expressed in the testis, small intestine, spleen, thymus, peripheral blood leukocytes, prostate, ovary and colon.

*SIVA* – Upregulation in CD27 and SIVA was demonstrated in renal dysfunction (e.g., ischemic and injured renal tissue). The expression of both proteins was seen in cell populations known to be undergoing death via apoptosis or necrosis [Schumer et al., Am. J. Pathol. 140:831-838, (1992); Shimzu and Yamanaka, Virchows Archiv. B Cell Pathol. 64:171-180; (1993), Basile et al., Am J. Physiol. 272: F640-F647, (1997)]. It was suggested that strategies directed at modifying CD27-mediated renal apoptosis will impact positively on the course of acute ischemic renal injury [Padanilam et al., Kidney Int. 54:1967-1975, (1998)]. Thus, the present invention envisages down-regulation of SIVA signaling through NIK-dependent NF- $\kappa$ B pathway to overcome the above-described renal disorders. SIVA interaction with the capsid protein VP2 of coxsackievirus B3 (CVB3) was shown to sustain CVB3-caused disease [Henke (2003) Clin. Exp. Med. 2(4):192-6]. Thus, the present invention envisages down-regulation of SIVA signaling through NIK-dependent NF- $\kappa$ B pathway to overcome viral disorders. Absence of SIVA-CD27 interactions was implicated in myelomagenesis, suggesting up-regulation of SIVA-CD27 signaling through NIK-dependent NF- $\kappa$ B pathway to overcome myelomagenesis [Katayama (2003) Br J Haematol. 120(2):223-34].

As mentioned hereinabove treating the immune disorder, according to the present invention, is effected by providing to the individual an agent which is capable

of increasing (i.e., upregulating) or decreasing (i.e., downregulating) the activity of at least one target gene or gene product, such as described hereinabove.

An agent capable of upregulating expression of a target gene of the present invention may be an exogenous polynucleotide sequence designed and constructed to  
5 express at least a functional portion of the target gene of the present invention. Accordingly, the exogenous polynucleotide sequence may be a DNA or RNA sequence encoding a CD27 (GenBank Accession No. NM\_001242), CD40L (GenBank Accession No. NM\_000074), BLys (GenBank Accession No. NM\_006573), SIVA (SIVA1 and SIVA2, GenBank Accession NO: NM\_006427 and  
10 NM\_021709, respectively), or NIK (GenBank Accession number NM\_003954) molecule, capable of modulating the immune disorder.

Thus, for example, to express exogenous NIK in mammalian cells, a polynucleotide sequence encoding NIK (SEQ ID NO:1) is preferably ligated into a nucleic acid construct suitable for mammalian cell expression. Such a nucleic acid  
15 construct includes a promoter sequence for directing transcription of the polynucleotide sequence in the cell in a constitutive or inducible manner. A suitable promoter can be, for example, a promoter derived from Lentiviral vectors (e.g., pSUPER) which is capable of directing NIK expression in B lymphocytes (see Example 2). The nucleic acid construct of the present invention can further include  
20 additional polynucleotide sequences such as for example, sequences encoding selection markers or reporter polypeptides, sequences encoding origin of replication in bacteria, sequences that allow for translation of several proteins from a single mRNA (IRES) such as for directing the simultaneous expression of NIK and SIVA to obtain higher expression levels of each and as such higher NF- $\kappa$ B activation levels (see  
25 Example 1 of the Examples section), sequences for genomic integration of the promoter-chimeric polypeptide encoding region and/or sequences generally included in mammalian expression vector such as pcDNA3, pcDNA3.1(+/-), pZeoSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, which are available from Invitrogen, pCI which is available from Promega, pBK-RSV and pBK-CMV  
30 which are available from Stratagene, pTRES which is available from Clontech, and their derivatives.

It will be appreciated that the nucleic acid construct can be administered to the individual employing any suitable mode of administration, such as described

hereinbelow (i.e., in-vivo gene therapy). Alternatively, the nucleic acid construct is introduced into a suitable cell via an appropriate gene delivery vehicle/method (transfection, transduction, homologous recombination, etc.) and an expression system as needed and then the modified cells are expanded in culture and returned to the individual (i.e., ex-vivo gene therapy).

Currently preferred in vivo nucleic acid transfer techniques include transfection with viral or non-viral constructs, such as adenovirus, lentivirus, Herpes simplex I virus, or adeno-associated virus (AAV) and lipid-based systems. Useful lipids for lipid-mediated transfer of the gene are, for example, DOTMA, DOPE, and DC-Chol [Tonkinson et al., Cancer Investigation, 14(1): 54-65 (1996)]. The most preferred constructs for use in gene therapy are viruses, most preferably adenoviruses, AAV, lentiviruses, or retroviruses. A viral construct such as a retroviral construct includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means such as alternate splicing, nuclear RNA export, or post-translational modification of messenger. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and positive and negative strand primer binding sites appropriate to the virus used, unless it is already present in the viral construct. In addition, such a construct typically includes a signal sequence for secretion of the peptide from a host cell in which it is placed. Preferably the signal sequence for this purpose is a mammalian signal sequence or the signal sequence of the polypeptide variants of the present invention. Optionally, the construct may also include a signal that directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof. Other vectors can be used that are non-viral, such as cationic lipids, polylysine, and dendrimers.

An agent capable of upregulating a target gene of the present invention may also be any compound which is capable of increasing the transcription and/or translation of an endogenous DNA or mRNA encoding the a target gene of the present invention. For example, PHA can be utilized to increase CD27 and CD70. In addition Anti-CD2 and Anti-CD3 antibodies can be utilized to increase CD27 levels (de Jong et al) while CD40L expression can be utilized to increase CD70 levels (Hintzen et al)

Alternatively or additionally, upregulation may be effected by administering to the individual at least one target such, as described hereinabove. Such proteins can be biochemically synthesized such as by using standard solid phase techniques. These methods include exclusive solid phase synthesis, partial solid phase synthesis methods, fragment condensation, classical solution synthesis. These methods are preferably used when the peptide is relatively short (i.e., 10 kDa) and/or when it cannot be produced by recombinant techniques (i.e., not encoded by a nucleic acid sequence) and therefore involves different chemistry.

Solid phase peptide synthesis procedures are well known in the art and further described by John Morrow Stewart and Janis Dillaha Young, Solid Phase Peptide Syntheses (2nd Ed., Pierce Chemical Company, 1984).

Synthetic peptides can be purified by preparative high performance liquid chromatography [Creighton T. (1983) Proteins, structures and molecular principles. WH Freeman and Co. N.Y.] and the composition of which can be confirmed via amino acid sequencing.

In cases where large amounts of the peptides of the present invention are desired, the proteins of the present invention can be generated using recombinant techniques such as described for the large scale production of recombinant CD70 in HEK293T cells (see Example 2 of the Examples section which follows) and by Bitter et al., (1987) Methods in Enzymol. 153:516-544, Studier et al. (1990) Methods in Enzymol. 185:60-89, Brisson et al. (1984) Nature 310:511-514, Takamatsu et al. (1987) EMBO J. 6:307-311, Coruzzi et al. (1984) EMBO J. 3:1671-1680 and Brogli et al., (1984) Science 224:838-843, Gurley et al. (1986) Mol. Cell. Biol. 6:559-565 and Weissbach & Weissbach, 1988, Methods for Plant Molecular Biology, Academic Press, NY, Section VIII, pp 421-463.

It will be appreciated that protein targets of the present invention can also be commercially obtained. For example, recombinant BAFF (Cat. No. PF088) and recombinant CD40L (Cat. No. PF091) are available from MERCK Biosciences.

As mentioned hereinabove, treatment of immune disorders according to the present invention can also be effected by down-regulating a target gene of product thereof, such as described hereinabove.

One example, of an agent capable of downregulating a target gene product of the present invention is an antibody or antibody fragment capable of specifically



binding the target gene product and inhibit binding thereof to effector molecules. For example, antibodies directed at amino acid coordinates 624-947 of NIK (SEQ ID NO: 2), at amino acid coordinates 123-175 of SIVA1 (SEQ ID NO:3) or at amino acid coordinates 58-110 of SIVA2 (SEQ ID NO:4) will prevent NIK-SIVA complex formation to thereby reduce NF- $\kappa$ B signaling. Alternatively, the antibodies of the present invention may still retain binding of the target gene product to effector molecules thereof but inhibit catalytic activity of thereof. Such an antibody directed against phosphorylated NIK activation loop is described in Example 4 of the Examples section which follows.

Preferably, the antibody specifically binds at least one epitope of the target gene product. As used herein, the term "epitope" refers to any antigenic determinant on an antigen to which the paratope of an antibody binds.

Epitopic determinants usually consist of chemically active surface groupings of molecules such as amino acids or carbohydrate side chains and usually have specific three dimensional structural characteristics, as well as specific charge characteristics.

The term "antibody" as used in this invention includes intact molecules as well as functional fragments thereof, such as Fab, F(ab')<sub>2</sub>, and Fv that are capable of binding to macrophages. These functional antibody fragments are defined as follows:

(1) Fab, the fragment which contains a monovalent antigen-binding fragment of an antibody molecule, can be produced by digestion of whole antibody with the enzyme papain to yield an intact light chain and a portion of one heavy chain; (2) Fab', the fragment of an antibody molecule that can be obtained by treating whole antibody with pepsin, followed by reduction, to yield an intact light chain and a portion of the heavy chain; two Fab' fragments are obtained per antibody molecule; (3) (Fab')<sub>2</sub>, the fragment of the antibody that can be obtained by treating whole antibody with the enzyme pepsin without subsequent reduction; F(ab')<sub>2</sub> is a dimer of two Fab' fragments held together by two disulfide bonds; (4) Fv, defined as a genetically engineered fragment containing the variable region of the light chain and the variable region of the heavy chain expressed as two chains; and (5) Single chain antibody ("SCA"), a genetically engineered molecule containing the variable region of the light chain and the variable region of the heavy chain, linked by a suitable polypeptide linker as a genetically fused single chain molecule.

Methods of producing polyclonal and monoclonal antibodies as well as fragments thereof are well known in the art (See for example, Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, New York, 1988, incorporated herein by reference).

5       Antibody fragments according to the present invention can be prepared by proteolytic hydrolysis of the antibody or by expression in *E. coli* or mammalian cells (e.g. Chinese hamster ovary cell culture or other protein expression systems) of DNA encoding the fragment. Antibody fragments can be obtained by pepsin or papain digestion of whole antibodies by conventional methods. For example, antibody  
10 fragments can be produced by enzymatic cleavage of antibodies with pepsin to provide a 5S fragment denoted F(ab')<sub>2</sub>. This fragment can be further cleaved using a thiol reducing agent, and optionally a blocking group for the sulfhydryl groups resulting from cleavage of disulfide linkages, to produce 3.5S Fab' monovalent fragments. Alternatively, an enzymatic cleavage using pepsin produces two  
15 monovalent Fab' fragments and an Fc fragment directly. These methods are described, for example, by Goldenberg, U.S. Pat. Nos. 4,036,945 and 4,331,647, and references contained therein, which patents are hereby incorporated by reference in their entirety. See also Porter, R. R. [Biochem. J. 73: 119-126 (1959)]. Other methods of cleaving antibodies, such as separation of heavy chains to form monovalent light-heavy chain  
20 fragments, further cleavage of fragments, or other enzymatic, chemical, or genetic techniques may also be used, so long as the fragments bind to the antigen that is recognized by the intact antibody.

Fv fragments comprise an association of VH and VL chains. This association may be noncovalent, as described in Inbar *et al.* [Proc. Nat'l Acad. Sci. USA 69:2659-  
25 62 (19720)]. Alternatively, the variable chains can be linked by an intermolecular disulfide bond or cross-linked by chemicals such as glutaraldehyde. Preferably, the Fv fragments comprise VH and VL chains connected by a peptide linker. These single-chain antigen binding proteins (sFv) are prepared by constructing a structural gene comprising DNA sequences encoding the VH and VL domains connected by an  
30 oligonucleotide. The structural gene is inserted into an expression vector, which is subsequently introduced into a host cell such as *E. coli*. The recombinant host cells synthesize a single polypeptide chain with a linker peptide bridging the two V domains. Methods for producing sFvs are described, for example, by [Whitlow and

Filpula, Methods 2: 97-105 (1991); Bird *et al.*, Science 242:423-426 (1988); Pack *et al.*, Bio/Technology 11:1271-77 (1993); and U.S. Pat. No. 4,946,778, which is hereby incorporated by reference in its entirety.

Another form of an antibody fragment is a peptide coding for a single complementarity-determining region (CDR). CDR peptides ("minimal recognition units") can be obtained by constructing genes encoding the CDR of an antibody of interest. Such genes are prepared, for example, by using the polymerase chain reaction to synthesize the variable region from RNA of antibody-producing cells. See, for example, Larrick and Fry [Methods, 2: 106-10 (1991)].

Humanized forms of non-human (e.g., murine) antibodies are chimeric molecules of immunoglobulins, immunoglobulin chains or fragments thereof (such as Fv, Fab, Fab', F(ab').sub.2 or other antigen-binding subsequences of antibodies) which contain minimal sequence derived from non-human immunoglobulin. Humanized antibodies include human immunoglobulins (recipient antibody) in which residues form a complementary determining region (CDR) of the recipient are replaced by residues from a CDR of a non-human species (donor antibody) such as mouse, rat or rabbit having the desired specificity, affinity and capacity. In some instances, Fv framework residues of the human immunoglobulin are replaced by corresponding non-human residues. Humanized antibodies may also comprise residues which are found neither in the recipient antibody nor in the imported CDR or framework sequences. In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the CDR regions correspond to those of a non-human immunoglobulin and all or substantially all of the FR regions are those of a human immunoglobulin consensus sequence. The humanized antibody optimally also will comprise at least a portion of an immunoglobulin constant region (Fc), typically that of a human immunoglobulin [Jones *et al.*, Nature, 321:522-525 (1986); Riechmann *et al.*, Nature, 332:323-329 (1988); and Presta, Curr. Op. Struct. Biol., 2:593-596 (1992)].

Methods for humanizing non-human antibodies are well known in the art. Generally, a humanized antibody has one or more amino acid residues introduced into it from a source which is non-human. These non-human amino acid residues are often referred to as import residues, which are typically taken from an import variable domain. Humanization can be essentially performed following the method of Winter

and co-workers [Jones *et al.*, Nature, 321:522-525 (1986); Riechmann *et al.*, Nature 332:323-327 (1988); Verhoeven *et al.*, Science, 239:1534-1536 (1988)], by substituting rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. Accordingly, such humanized antibodies are chimeric antibodies (U.S. Pat. No. 4,816,567), wherein substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some FR residues are substituted by residues from analogous sites in rodent antibodies.

Human antibodies can also be produced using various techniques known in the art, including phage display libraries [Hoogenboom and Winter, J. Mol. Biol., 227:381 (1991); Marks *et al.*, J. Mol. Biol., 222:581 (1991)]. The techniques of Cole *et al.* and Boerner *et al.* are also available for the preparation of human monoclonal antibodies (Cole *et al.*, Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, p. 77 (1985) and Boerner *et al.*, J. Immunol., 147(1):86-95 (1991)]. Similarly, human antibodies can be made by introduction of human immunoglobulin loci into transgenic animals, e.g., mice in which the endogenous immunoglobulin genes have been partially or completely inactivated. Upon challenge, human antibody production is observed, which closely resembles that seen in humans in all respects, including gene rearrangement, assembly, and antibody repertoire. This approach is described, for example, in U.S. Pat. Nos. 5,545,807; 5,545,806; 5,569,825; 5,625,126; 5,633,425; 5,661,016, and in the following scientific publications: Marks *et al.*, Bio/Technology 10: 779-783 (1992); Lonberg *et al.*, Nature 368: 856-859 (1994); Morrison, Nature 368 812-13 (1994); Fishwild *et al.*, Nature Biotechnology 14, 845-51 (1996); Neuberger, Nature Biotechnology 14: 826 (1996); and Lonberg and Huszar, Intern. Rev. Immunol. 13, 65-93 (1995).

Another agent capable of downregulating a target gene of the present invention is a small interfering RNA (siRNA) molecule.

RNA interference is a two step process; the first step, which is termed as the initiation step, input dsRNA is digested into 21-23 nucleotide (nt) small interfering RNAs (siRNA), probably by the action of Dicer, a member of the RNase III family of dsRNA-specific ribonucleases, which processes (cleaves) dsRNA (introduced directly or via a transgene or a virus) in an ATP-dependent manner. Successive cleavage

events degrade the RNA to 19-21 bp duplexes (siRNA), each with 2-nucleotide 3' overhangs [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002); and Bernstein Nature 409:363-366 (2001)].

In the effector step, the siRNA duplexes bind to a nuclease complex to from  
5 the RNA-induced silencing complex (RISC). An ATP-dependent unwinding of the siRNA duplex is required for activation of the RISC. The active RISC then targets the homologous transcript by base pairing interactions and cleaves the mRNA into 12 nucleotide fragments from the 3' terminus of the siRNA [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002); Hammond *et al.* (2001)  
10 Nat. Rev. Gen. 2:110-119 (2001); and Sharp Genes. Dev. 15:485-90 (2001)]. Although the mechanism of cleavage is still to be elucidated, research indicates that each RISC contains a single siRNA and an RNase [Hutvagner and Zamore Curr. Opin. Genetics and Development 12:225-232 (2002)].

Because of the remarkable potency of RNAi, an amplification step within the  
15 RNAi pathway has been suggested. Amplification could occur by copying of the input dsRNAs which would generate more siRNAs, or by replication of the siRNAs formed. Alternatively or additionally, amplification could be effected by multiple turnover events of the RISC [Hammond *et al.* Nat. Rev. Gen. 2:110-119 (2001), Sharp Genes. Dev. 15:485-90 (2001); Hutvagner and Zamore Curr. Opin. Genetics and  
20 Development 12:225-232 (2002)]. For more information on RNAi see the following reviews Tuschl ChemBiochem. 2:239-245 (2001); Cullen Nat. Immunol. 3:597-599 (2002); and Brantl Biochem. Biophys. Act. 1575:15-25 (2002).

Synthesis of RNAi molecules suitable for use with the present invention can be effected as follows. First, the mRNA sequence is scanned downstream of the AUG  
25 start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA  
30 endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90

% decrease in cellular GAPDH mRNA and completely abolished protein level ([www.ambion.com/techlib/tn/91/912.html](http://www.ambion.com/techlib/tn/91/912.html)).

Second, potential target sites are compared to an appropriate genomic database (e.g., human, mouse, rat etc.) using any sequence alignment software, such as the BLAST software available from the NCBI server ([www.ncbi.nlm.nih.gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation.

An siRNA molecule capable of specifically hybridizing with the mRNA of NIK to arrest synthesis thereof is described in Example 2 of the Examples section which follows (SEQ ID NO:15).

For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

Another agent capable of downregulating a target gene of the present invention is a DNzyme molecule capable of specifically cleaving an mRNA transcript or DNA sequence of the target gene of the present invention. DNzymes are single-stranded polynucleotides which are capable of cleaving both single and double stranded target sequences (Breaker, R.R. and Joyce, G. Chemistry and Biology 1995;2:655; Santoro, S.W. & Joyce, G.F. Proc. Natl. Acad. Sci. USA 1997;94:4262). A general model (the "10-23" model) for the DNzyme has been proposed. "10-23" DNzymes have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. This type of DNzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions (Santoro, S.W. & Joyce, G.F. Proc. Natl. Acad. Sci. USA 1997; for rev of DNzymes see Khachigian, LM [Curr Opin Mol Ther 4:119-21 (2002)]).

Examples of construction and amplification of synthetic, engineered DNazymes recognizing single and double-stranded target cleavage sites have been disclosed in U.S. Pat. No. 6,326,174 to Joyce *et al.* DNazymes of similar design directed against the human Urokinase receptor were recently observed to inhibit Urokinase receptor expression, and successfully inhibit colon cancer cell metastasis in vivo (Itoh *et al.*, 20002, Abstract 409, Ann Meeting Am Soc Gen Ther [www.asgt.org](http://www.asgt.org)). In another application, DNazymes complementary to bcr-abl oncogenes were successful in inhibiting the oncogenes expression in leukemia cells, and lessening relapse rates in autologous bone marrow transplant in cases of CML and ALL.

Downregulation of a target gene of the present invention can also be effected by using an antisense polynucleotide capable of specifically hybridizing with an mRNA transcript encoding the target gene product.

Design of antisense molecules which can be used to efficiently downregulate a target gene must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically binds the designated mRNA within cells in a way which inhibits translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Luft J Mol Med 76: 75-6 (1998); Kronenwett *et al.* Blood 91: 852-62 (1998); Rajur *et al.* Bioconjug Chem 8: 935-40 (1997); Lavigne *et al.* Biochem Biophys Res Commun 237: 566-71 (1997) and Aoki *et al.* (1997) Biochem Biophys Res Commun 231: 540-5 (1997)].

In addition, algorithms for identifying these sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton *et al.* Biotechnol Bioeng 65: 1-9 (1999)].

Such algorithms have been successfully used to implement an antisense approach in cells. For example, the algorithm developed by Walton *et al.* enabled scientists to successfully design antisense oligonucleotides for rabbit beta-globin

(RBG) and mouse tumor necrosis factor-alpha (TNF alpha) transcripts. The same research group has more recently reported that the antisense activity of rationally selected oligonucleotides against three model target mRNAs (human lactate dehydrogenase A and B and rat gp130) in cell culture as evaluated by a kinetic PCR  
5 technique proved effective in almost all cases, including tests against three different targets in two cell types with phosphodiester and phosphorothioate oligonucleotide chemistries.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an *in vitro* system were also published (Matveeva *et al.*, Nature Biotechnology 16: 1374 - 1375 (1998)).  
10

Several clinical trials have demonstrated safety, feasibility and activity of antisense oligonucleotides. For example, antisense oligonucleotides suitable for the treatment of cancer have been successfully used [Holmund *et al.*, Curr Opin Mol Ther 1:372-85 (1999)], while treatment of hematological malignancies via antisense  
15 oligonucleotides targeting c-myc gene, p53 and Bcl-2 had entered clinical trials and had been shown to be tolerated by patients [Gerwitz Curr Opin Mol Ther 1:297-306 (1999)].

More recently, antisense-mediated suppression of human heparanase gene expression has been reported to inhibit pleural dissemination of human cancer cells in  
20 a mouse model [Uno *et al.*, Cancer Res 61:7855-60 (2001)].

Thus, the current consensus is that recent developments in the field of antisense technology which, as described above, have led to the generation of highly accurate antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily skilled artisan to design and implement antisense  
25 approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation.

Another agent capable of downregulating a target gene of the present invention is a ribozyme molecule capable of specifically cleaving an mRNA transcript encoding a target gene product. Ribozymes are being increasingly used for the  
30 sequence-specific inhibition of gene expression by the cleavage of mRNAs encoding proteins of interest [Welch *et al.*, Curr Opin Biotechnol. 9:486-96 (1998)]. The possibility of designing ribozymes to cleave any specific target RNA has rendered them valuable tools in both basic research and therapeutic applications. In the



therapeutics area, ribozymes have been exploited to target viral RNAs in infectious diseases, dominant oncogenes in cancers and specific somatic mutations in genetic disorders [Welch *et al.*, Clin Diagn Virol. 10:163-71 (1998)]. Most notably, several ribozyme gene therapy protocols for HIV patients are already in Phase 1 trials. More recently, ribozymes have been used for transgenic animal research, gene target validation and pathway elucidation. Several ribozymes are in various stages of clinical trials. ANGIOZYME was the first chemically synthesized ribozyme to be studied in human clinical trials. ANGIOZYME specifically inhibits formation of the VEGF-r (Vascular Endothelial Growth Factor receptor), a key component in the angiogenesis pathway. Ribozyme Pharmaceuticals, Inc., as well as other firms have demonstrated the importance of anti-angiogenesis therapeutics in animal models. HEPTAZYME, a ribozyme designed to selectively destroy Hepatitis C Virus (HCV) RNA, was found effective in decreasing Hepatitis C viral RNA in cell culture assays (Ribozyme Pharmaceuticals, Incorporated - WEB home page).

An additional method of regulating the expression of a target gene in cells is via triplex forming oligonucleotides (TFOs). Recent studies have shown that TFOs can be designed which can recognize and bind to polypurine/polypirimidine regions in double-stranded helical DNA in a sequence-specific manner. These recognition rules are outlined by Maher III, L. J., et al., Science, 1989;245:725-730; Moser, H. E., et al., Science, 1987;238:645-630; Beal, P. A., et al., Science, 1992;251:1360-1363; Cooney, M., et al., Science, 1988;241:456-459; and Hogan, M. E., et al., EP Publication 375408. Modification of the oligonucleotides, such as the introduction of intercalators and backbone substitutions, and optimization of binding conditions (pH and cation concentration) have aided in overcoming inherent obstacles to TFO activity such as charge repulsion and instability, and it was recently shown that synthetic oligonucleotides can be targeted to specific sequences (for a recent review see Seidman and Glazer, J Clin Invest 2003;112:487-94).

In general, the triplex-forming oligonucleotide has the sequence correspondence:

oligo	3'--A	G	G	T
duplex	5'--A	G	C	T
duplex	3'--T	C	G	A

However, it has been shown that the A-AT and G-GC triplets have the greatest

triple helical stability (Reither and Jeltsch, BMC Biochem, 2002, Sept12, Epub). The same authors have demonstrated that TFOs designed according to the A-AT and G-GC rule do not form non-specific triplexes, indicating that the triplex formation is indeed sequence specific.

5 Thus for any given sequence in the target gene regulatory region a triplex forming sequence may be devised. Triplex-forming oligonucleotides preferably are at least 15, more preferably 25, still more preferably 30 or more nucleotides in length, up to 50 or 100 bp.

10 Transfection of cells (for example, via cationic liposomes) with TFOs, and formation of the triple helical structure with the target DNA induces steric and functional changes, blocking transcription initiation and elongation, allowing the introduction of desired sequence changes in the endogenous DNA and resulting in the specific downregulation of gene expression. Examples of such suppression of gene expression in cells treated with TFOs include knockout of episomal *supFGL* and  
15 endogenous *HPRT* genes in mammalian cells (Vasquez et al., Nucl Acids Res. 1999;27:1176-81, and Puri, et al, J. Biol Chem, 2001;276:28991-98), and the sequence- and target specific downregulation of expression of the Ets2 transcription factor, important in prostate cancer etiology (Carbone, et al, Nucl Acid Res. 2003;31:833-43), and the pro-inflammatory ICAM-1 gene (Besch et al, J Biol Chem,  
20 2002;277:32473-79). In addition, Vuyisich and Beal have recently shown that sequence specific TFOs can bind to dsRNA, inhibiting activity of dsRNA-dependent enzymes such as RNA-dependent kinases (Vuyisich and Beal, Nuc. Acids Res 2000;28:2369-74).

25 Additionally, TFOs designed according to the abovementioned principles can induce directed mutagenesis capable of effecting DNA repair, thus providing both downregulation and upregulation of expression of endogenous genes (Seidman and Glazer, J Clin Invest 2003;112:487-94). Detailed description of the design, synthesis and administration of effective TFOs can be found in U.S. Patent Application Nos. 2003 017068 and 2003 0096980 to Froehler et al, and 2002 0128218 and 2002  
30 0123476 to Emanuele et al, and U.S. Pat. No. 5,721,138 to Lawn.

It will be appreciated that polynucleotides and polypeptides such as described hereinabove, can also be used to downregulate an activity of a target gene product.

Thus, for example, a NIK polypeptide or polynucleotide encoding same which includes the mouse naturally-occurring mutation, alymphoplasia (aly), can be used to down-regulate NIK dependent NF- $\kappa$ B signaling. This mutation is autosomal recessive and was shown to result in the systemic absence of lymph nodes and Peyer patches, disorganized splenic and thymic structures with immunodeficiency in mice carrying thereof [Shinkura (1999) *Nature Genet.* 22:74-77].

It will be appreciated that down-regulating polypeptides and polynucleotides encoding same can be characterized by a dominant negative function, essentially a dominant effect on the activity of the wild type target gene product. For example, a protein product of kinase defective NIK may bind effector proteins (e.g., SIVA), thus forming inactive complexes and inhibiting NIK dependent NF- $\kappa$ B signaling. Dominant negative mutants of NIK are well known in the art, see for example Hay (2003) *Biochem. Biophys. Acta.* 1642(1-2):33-44; and Chandrasekar (2003) *Biochem. J.* 373:547-58. A dominant negative molecule of IKK $\alpha$  which blocks NF- $\kappa$ B activation is described by Shikama (2003) *Eur. J. Immunol.* 33:1998-2006. A dominant negative molecule of TRAF-2 is described by Costabanzo (2003) *J. Cell Physiol.* 195:402-10.

Alternatively, an agent capable of downregulating a target gene of the present invention may also be any compound which is capable of decreasing the transcription and/or translation process or levels of an endogenous DNA or mRNA encoding the a target gene product of the present invention. For example, PMA treatment may be used to CD27 mRNA levels as described in De Jonge 1991 *infra*.

It will be appreciated that additional agents (i.e., putative immune modulators) which may be used in the present invention can be identified by examining the ability thereof to increase or decrease NIK-dependent NF- $\kappa$ B signaling. Thus, for example, agents may be tested for their ability to increase or decrease NIK-SIVA complex formation, or NIK-dependent CD27 regulation, using cytological, genetical and/or biochemical methods which are well known in the art and described in Examples 1-3 of the Examples section, which follows.

Molecules/agents which can be used to modulate the activity of NIK may be screened (identified and/or selected) by contacting a cell with a ligand of a TNF/NGF receptor family capable to induce NIK-dependent canonical and alternative pathway in the cell, incubating the cell prior to, after, or during said contacting with

individual tested molecules, detecting activation of the canonical pathway in the cell and selecting individual molecule/s capable of modulating induction of the canonical pathway induced by said ligand.

In a preferred embodiment, CD70, CD40L, or Blys/BAFF ligand is used for the screening of molecules. Alternatively, new ligands capable to induce NIK dependent canonical and alternative pathway can be identified, as exemplified below for CD70.

The detection of canonical pathway activation, for the screening of the molecules, can be carried out by monitoring parameters indicative of the canonical pathway such as I $\kappa$ B degradation, I $\kappa$ B $\alpha$  phosphorylation and p65 translocation.

In a preferred embodiment of the invention, lymphoblastoid cell type such as Ramos, Raji and BJAB cells are used for the screening of the molecules of the invention..

In addition molecules/agents which can be used to modulate the activity of NIK may be screened by contacting a lymphoblastoid cell with a ligand of a TNF/NGF receptor family capable of activating NIK and the canonical pathway in the cell, incubating the cell prior to, after, or during said contacting, with individual tested molecules, detecting activation of the canonical pathway and, selecting individual molecule/s capable of modulating induction of the canonical pathway induced by said ligand but not by any other ligand capable of inducing canonical pathway in a NIK independent manner, such as TNF.

The agents of the present invention can be administered to the subject *per se*, or as part of a pharmaceutical composition where they are mixed with a pharmaceutically acceptable carrier.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to the preparation accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An  
5 adjuvant is included under these phrases. One of the ingredients included in the pharmaceutically acceptable carrier can be for example polyethylene glycol (PEG), a biocompatible polymer with a wide range of solubility in both organic and aqueous media (Mutter et al. (1979).

Herein the term "excipient" refers to an inert substance added to a  
10 pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in  
15 "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct  
20 intraventricular, intravenous, intraperitoneal, intranasal, or intraocular injections. Alternately, one may administer a preparation in a local rather than systemic manner, for example, via injection of the preparation directly into a specific region of a patient's body.

Pharmaceutical compositions of the present invention may be manufactured by  
25 processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with the present invention may be formulated in conventional manner using one or more physiologically  
30 acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

For oral administration, the compounds can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the compounds of the invention to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carbomethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions, which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added.

All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

5 For administration by nasal inhalation, the active ingredients for use according to the present invention are conveniently delivered in the form of an aerosol spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichloro-  
10 tetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The preparations described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for  
15 injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous  
20 solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of  
25 the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution  
30 with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The preparation of the present invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of the present invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients effective to prevent, alleviate or ameliorate symptoms of disease or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from *in vitro* assays. For example, a dose can be formulated in animal models and such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures *in vitro*, in cell cultures or experimental animals. The data obtained from these *in vitro* and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.1).

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions including the preparation of the present invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

Pharmaceutical compositions of the present invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or



dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination.

Additional objects, advantages, and novel features of the present invention will become apparent to one ordinarily skilled in the art upon examination of the following examples, which are not intended to be limiting. Additionally, each of the various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below finds experimental support in the following examples.

### EXAMPLES

Reference is now made to the following examples, which together with the above descriptions, illustrate the invention in a non limiting fashion.

Generally, the nomenclature used herein and the laboratory procedures utilized in the present invention include molecular, biochemical, microbiological and recombinant DNA techniques. Such techniques are thoroughly explained in the literature. See, for example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore, Maryland (1989); Perbal, "A Practical Guide to Molecular Cloning", John Wiley & Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory

Manual Series", Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057; "Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994); "Current Protocols in Immunology" Volumes I-III Coligan J. E., ed. (1994); Stites et al. (eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT (1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H. Freeman and Co., New York (1980); available immunoassays are extensively described in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture" Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A Practical Guide to Molecular Cloning" Perbal, B., (1984) and "Methods in Enzymology" Vol. 1-317, Academic Press; "PCR Protocols: A Guide To Methods And Applications", Academic Press, San Diego, CA (1990); Marshak et al., "Strategies for Protein Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996); all of which are incorporated by reference as if fully set forth herein. Other general references are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

### EXAMPLE 1

#### NIK-SIVA BINDING

The binding of NIK to SIVA and the effect of their co-expression were tested using binding, co-expression and immunoprecipitation assays.

#### *Antibodies:*

Anti-HIS antibody was purchased from Sigma. Anti-myc monoclonal antibody (clone-9E10) was purified from mouse ascitic fluid on a myc-peptide affinity column.

#### *Cells:*

HEK293T cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10 % fetal calf serum, 100 U/ml penicillin and 100 µg/ml streptomycin.

***Expression vectors:***

5 A vector for expressing NIK (SEQ ID NO:1) N-terminally fused to the myc tag (EQKLISEEDL, SEQ ID NO:5) was obtained from Dr. Michael Kracht, Germany. The cDNAs for human SIVA1 (SEQ ID NO:6) and SIVA2 (SEQ ID NO:7) were PCR-amplified from ESTs and cloned into the pcDNA3.1-HIS vector (Invitrogen). pEGFP plasmid was purchased from Clontech. Human NIK with a mutation  
10 corresponding to that of the mouse *aly* mutation (G860R) (Shinkura et al., 1999; NM\_016896) was generated with a site-directed mutagenesis kit (Stratagene), using (sense) 5' CCAAGCTATTTCAATCGTGTGAAAGTCCAAATAC (SEQ ID NO:8) and (antisense) 5' GTATTTGGACTTTCACACGATTGAAATAGCTTGG (SEQ ID NO:9).

15 ***Yeast two-hybrid screening:***

A BamHI/XhoI digested NIK insert from the pcNIK vector was subcloned into BamHI/SalI sites of the Gal4 DNA-binding domain vector pGBKT7 (Clontech). A pre-transformed human bone marrow library (HL4053AH, Clontech) was subjected to two-hybrid screening using pGBKT7-NIK as the bait, according to the manufacturer's  
20 instructions (Yeast protocol handbook, Clontech - [www.clontech.com](http://www.clontech.com)) Positive clones were identified by quadruple selection and β-galactosidase activity assay. Binding of the SIVA clone to NIK and NIK624-947 was reconfirmed and binding of NIK to TRAF2 was assessed by a β-galactosidase expression assay using the yeast SFY526 reporter strain (Clontech) and the pGBKT7 and pGADT7 vectors.

25 ***Transfections, immunoblotting and immunoprecipitations:***

For co-immunoprecipitation of transfected proteins, HEK293T cells were seeded onto 90-mm plates ( $1.5 \times 10^6$  cells/plate) and transfected using the calcium phosphate precipitation method (Sambrook et al., 1989) a day later using a total  
30 amount of 10 µg DNA in 10ml of DMEM medium with 10% FBS. For co-transfection a 1:1 mixture of the plasmids encoding tested proteins was used. Twenty four hours following transfection the cells were rinsed once with phosphate buffered saline (PBS) and lysed in 1 ml of lysis buffer (10 mM Tris-HCl (pH 7.6), 250 mM

NaCl, 1% NP-40, 1 mM EDTA, 1 mM PMSF) which included 1× complete protease inhibitor cocktail (Roche Molecular Biochemicals). Pre-cleared lysates were incubated for 2 hours at 4 °C with 2 µg of anti-myc or anti-HIS antibody preadsorbed to protein-G-Sepharose beads (Amersham biosciences). The beads were then rinsed  
5 with lysis buffer, subjected to SDS-PAGE, and the proteins were transferred to a nitrocellulose membrane and probed with the indicated antibodies. The antibodies were visualized with horseradish peroxidase (HRP)-coupled secondary antibodies, using the enhanced chemiluminescence (ECL) western blotting detection system (Amersham) according to the manufacturer's instructions.

10

***Reporter gene test:***

NIK-mediated NF-κB activation was measured by reporter gene assay. HEK293T cells ( $1.5 \times 10^5$ /well) were seeded onto 6-well plates and transfected by the  
15 calcium phosphate precipitation method (Sambrook et al., 1989) one day later. For co-transfection, a 1:1 mixture of the plasmids encoding the tested proteins was used. To maintain total DNA concentration at 2 µg/well, a pcDNA3 (Invitrogen) 'empty' vector was added. Twenty-four hours following transfection, the cells were harvested, lysed, and reporter gene activity was determined using the luciferase assay system  
20 (Promega).

***NIK binds to SIVA, an adapter protein associated with CD27:***

Screening of a human bone marrow two-hybrid library using NIK as bait uncovered that NIK specifically binds a C-terminal portion of a protein termed SIVA  
25 which was previously shown to associate with CD27, a receptor of the TNF/NGF family expressed mainly in T and B lymphocytes (Prasad et al., 1997). As with binding of TRAF to NIK (Malinin et al., 1997), the present study demonstrated that the C-terminal portion of SIVA binds the C-terminal portion of NIK and that this binding was stronger when the respective portions and not the whole protein was  
30 utilized in the binding assays (Figure 1a). This is probably due to the propensity of the N-terminal part of NIK to bind to its own C-terminus and thus block binding of this portion with other proteins (Xiao and Sun, 2000).

Co-expression of NIK with SIVA1 or SIVA2, the two known SIVA splice variants (Yoon et al., 1999), in transiently transfected HEK293 cells revealed that NIK can bind SIVA in mammalian cells. As shown in Figures 1b-c, NIK co-immunoprecipitated bidirectionally with both splice variants from lysates of the transfected cells. The quantity of SIVA1 or SIVA2 in the transfected cells increased when co-expressed with NIK, apparently reflecting stabilization of SIVA by its associated NIK molecules. The expression of NIK was also enhanced by the co-expression of either of the two SIVA splice variants (Figures 1b, 1c). No such enhancement was observed upon co-expression of NIK with green fluorescent protein (GFP) or IKK1 (Figure 1d). Expression of SIVA2 did not increase the quantity of co-expressed NIK containing an inactivating missense mutation corresponding to that found in the *aly* mice (G860R), even though this NIK mutant did co-immunoprecipitate with SIVA1 and to some extent also with SIVA2 (Figures 1b-c).

SIVA also appeared to be capable of affecting NIK function. When expressed alone, SIVA1 or SIVA2 caused only slight activation of NF- $\kappa$ B. However, both splice variants significantly enhanced the activation of NF- $\kappa$ B by co-expressed NIK while not affecting the activation of NF- $\kappa$ B by the co-expressed NIK *aly* mutant (Figure 1e).

## EXAMPLE 2

### ***CD27 INDUCES PROCESSING OF BOTH I $\kappa$ B AND NF- $\kappa$ B2/P100 IN LYMPHOCYTES, WHILE ARREST OF NIK SYNTHESIS IN RAMOS LYMPHOBLASTOID CELLS BLOCKS CD27-INDUCED NF- $\kappa$ B ACTIVATION***

The ability of CD70 (CD27 ligand) to induce processing of both I $\kappa$ B and NF- $\kappa$ B2/p100 in lymphocytes was determined via western blot analysis which utilized antibodies directed against p100, p52, RelB, I $\kappa$ B $\alpha$  and p65. The effect of the presence of NIK in these cells on the induced processing was assessed by western blot analysis of the same molecules on NIK suppressed cells.

#### ***Reagents:***

CD70 (GenBank Accession number Y13636) was produced by large-scale transfection of human embryonic kidney (HEK) 293T cells with the relevant expression constructs (see below). MG132 was purchased from Calbiochem. Ficoll-Paque was purchased from Amersham Biosciences. G418 was purchased from Life

Technologies. Phytohemagglutinin (PHA) was purchased from Sigma. In all tests, the conditioned medium of the transfected cells was used at a dilution of 1:4.

#### ***Antibodies:***

Anti-p52 antibody was purchased from Upstate Biotechnologies, antibodies  
 5 against p65, RelB, CD27 and Lamin A/C from Santa Cruz Biotechnology, anti-I $\kappa$ B $\alpha$   
 from Transduction Laboratories, anti-myc monoclonal antibody was purified as  
 described in Example 1. The anti-NIK monoclonal antibody NIK-81 was raised by  
 immunizing mice with a KLH-coupled peptide corresponding to a sequence within the  
 NIK kinase domain (RLGRGSFGEVHRMEDK, SEQ ID NO:10) which included a  
 10 cysteine at the N terminus for coupling. Anti-NIK monoclonal antibody was purified  
 on affigel (BIORAD), an affinity column to which the BSA linked peptide was  
 coupled.

#### ***Cells:***

Human peripheral blood mononuclear cells (PBMC) were isolated from buffy  
 15 coat samples by Ficoll-Paque gradient centrifugation at 450  $\times$  g. Cells were subjected  
 to stimulation with CD70 without any pretreatment or following their activation for  
 48 hours with 1  $\mu$ g/ml PHA that was followed by a 12 h resting period without PHA.  
 The PBMC, as well as cells of the human B lymphoblastoid lines of Burkitt  
 lymphoma origin, Ramos (Benjamin et al., 1982), Raji (Pulvertaft, 1964), were  
 20 cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml  
 penicillin and 100  $\mu$ g/ml streptomycin. HEK293T cells were cultured as described in  
 Example 1 of the Examples section.

#### ***Expression vectors:***

The cDNAs for the extracellular domains of mCD70, was PCR-amplified from  
 25 ESTs and cloned in fusion with a modified leucine zipper and FLAG tag (Fanslow et  
 al., 1994) into pcDNA3 (Invitrogen). The cDNA corresponding to the full-length NIK  
 was cloned into the Gal4 DNA-binding domain vector pGBKT7 (Clontech). NIK  
 which sequence was altered to make it non-complementary to the NIK siRNA, was  
 generated using (sense)  
 30 5'GAGGGTCTGGAATACCTACATTCCCGCAGGATTCTGCATGGG (SEQ ID  
 NO:11) and (antisense)  
 5'CCCATGCAGAATCCTGCGGGAATGTAGGTATTCCAGACCCTC (SEQ ID  
 NO:12), as primers.

***siRNA and lentiviral transduction:***

Hairpin siRNA was expressed using the pSUPER vector, as previously described (Brummelkamp et al., 2002). Briefly, a double-stranded oligonucleotide was designed to contain the forward and reverse sequences corresponding to a region in the human NIK open reading frame (nucleotides 1513–1531) linked by a 9-base-pair spacer region (ttcaagaga SEQ ID NO:15): sense strand 5' gatccccTACCTCCACTCACGAAGGAttcaagagaTCCTTCGTGAGTGGAGGTAttttg gaaa (SEQ ID NO:13); antisense strand 5' agcttttccaaaaTACCTCCACTCACGAAGGAtctcttgaaTCCTTCGTGAGTGGAGG TAggg (SEQ ID NO:14). The two oligonucleotides were annealed and cloned into the *Bgl*III and *Hind*III (Brummelkamp et al., 2002) sites of the pSUPER vector for expression under the control of the H1 RNA promoter (Brummelkamp et al., 2002). Transient transfection with up to 5-fold excess of this pSUPER-NIK over co-transfected NIK was performed, as described above.

A lentiviral vector (Lois et al., 2002) was used in order to express the pSUPER-NIK constitutively in Ramos cells. The cassette including the H1 promoter (Brummelkamp et al., 2002) and NIK RNAi was excised from the pSUPER vector using *Eco*RI and *Hind*III (both from New England Biolabs), the sticky ends were blunted using T4 DNA polymerase (New England Biolabs), and the blunted fragment was inserted into the blunted *Pac*I site of the GFP-expressing FUGW lentiviral vector (Lois et al., 2002). Transduced cells were sorted by FACS for GFP expression (FACS Vantage, Becton-Dickinson). Sorted cells exhibited expression of GFP and deficiency of NIK for months.

***Transfections, immunoblotting and immunoprecipitations:***

Transfections, immunoblotting and immunoprecipitations were performed as described in Example 1 of the Examples section.

Ramos cells ( $2-4 \times 10^8$ ;  $1 \times 10^8$  cells/ml) were lysed and immunoprecipitated with affinity-purified mouse anti-NIK antiserum coupled to protein-G–Sephadex beads in order to detect endogenous NIK. The precipitated protein was detected by western blotting using the NIK-81 antibody and the SuperSignal West Femto Chemiluminescent Detection Kit (Pierce).

For immunoprecipitation of the NF- $\kappa$ B proteins, nuclear extracts from  $10-20 \times 10^6$  cells were diluted to achieve the following composition: 0.5% NP-40, 10 mM

HEPES pH 7.9, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol (DTT), 1 mM PMSF, and 1× complete protease inhibitor cocktail. Anti-RelB antibody preadsorbed to protein-A-Sepharose beads (Amersham Biosciences) was incubated with precleared nuclear lysates for 2 hours at 4 °C. The immunoprecipitates  
5 were analyzed by SDS-PAGE using 4–12% Bis-Tris NuPAGE gels (Invitrogen), and the gels were subjected to western blotting as described above.

Ligand activation of lymphoid cell lines and PBMC, was typically carried by stimulating  $1 \times 10^6$  cells for the time periods indicated in the figures (0, 0.3 and 4 hours or 0, 1 and 4 hours; Figure 2a-b) with the relevant ligands, and nuclear and  
10 cytoplasmic extracts were prepared as described (Schreiber et al., 1989) and analyzed by western blotting.

Ligand activation of Raji cells was carried by stimulating cells for the time periods indicated in the figures (0, 0.3 and 4 hours or 0, 1 and 4 hours; Figure 2a-b) with CD70. Ligand activation of normal and NIK<sup>(MINUS)</sup> Ramos cells was carried by  
15 stimulating cells for the time periods indicated in the figures (0, 0.3, 1, 4 and 20 hours; Figures 2d-e or 0, 0.15, 0.3, 4 and 16 hours; Figure 2h) with CD70.

Reintroduction of NIK to the NIK<sup>(MINUS)</sup> cells was performed by nucleotransfection of NIK<sup>(MINUS)</sup> cells with the expressing vector using the Nucleofector Kit V according to the manufacturer's instructions (Amaxa Biosystems).  
20 Briefly,  $2 \times 10^6$  NIK<sup>(MINUS)</sup> cells were nucleofected with 4 µg of mutated myc-tagged NIK plasmid and 1 µg of pcDNA3 in solution V using the program S18 in the Nucleofector device. Cells stably expressing the transfected protein were selected on G418 (1 mg/ml).

#### ***Protein kinase C assay:***

25 PKC activity in control and NIK deficient Ramos cells was measured (0, 15 and 30 minutes) following CD70 stimulation by using the Signatect Protein Kinase C assay system (Promega). The enzymatic activity of PKC was determined by subtracting the values obtained when assaying in the absence of phospholipids from those obtained in their presence.



***CD27 induces processing of both I $\kappa$ B and NF- $\kappa$ B2/p100 in lymphocytes:***

The ability of NIK to bind to SIVA suggested that NIK might play a role in the cellular function of CD27. Therefore, the effects of CD27 on the alternative NF- $\kappa$ B activation pathway in which NIK function has been implicated was examined.

5 Treatment of human peripheral blood mononuclear leukocytes (PBMC) with the CD27 ligand, CD70, induced rapid decrease of I $\kappa$ B $\alpha$  (Figures 2a, 2b, top panels), indicating that the receptor can trigger activation of the canonical NF- $\kappa$ B pathway. This decrease was easier to detect in activated PBMC, in which the basal I $\kappa$ B $\alpha$  level is high (Figure 2b, top panel), but on careful examination it could also be discerned in  
10 non-stimulated PBMC, which contain much less I $\kappa$ B $\alpha$  (Figure 2a, top panel). In resting PBMCs, CD70 also induced translocation of NF- $\kappa$ B2/p52 (p52) as well as of RelB to the nuclei, indicating that CD27 stimulates the alternative NF- $\kappa$ B pathway in these cells as well (Figure 2a, bottom panel). Following PHA activation, which results in increased expression of both CD27 and CD70 in PBMC (de Jong et al., 1991 and  
15 Hintzen et al., 1994), the basal nuclear level of both p52 and RelB was very high, preventing the assessment of the effect of applied CD70 on p52 generation (Figure 2b, bottom panel).

The present study also showed that CD70 induces I $\kappa$ B $\alpha$  degradation as well as nuclear translocation of RelB and NF- $\kappa$ B2/p52 in Ramos and Raji lymphoblastoid  
20 lines (Figure 2c and left panels in Figure 2d, 2e). Treatment of Raji cells with CD70 induces I $\kappa$ B $\alpha$  degradation within 20 minutes of CD70 application while later on I $\kappa$ B $\alpha$  levels increased. I $\kappa$ B $\alpha$  degradation was associated with nuclear translocation of p65, RelB and NF- $\kappa$ B2/p52 (Figure 2c).

Similar pattern of I $\kappa$ B $\alpha$  degradation was observed in Ramos cells following  
25 CD70 application (Figure 2e, left panel). I $\kappa$ B $\alpha$  degradation was associated with a prolonged increase in nuclear p65 levels (Figure 2e, left panel). On the other hand, the nuclear translocation of RelB occurred rather slowly, reaching maximal translocation about 4 hours following CD70 application (Figure 2d, left panel). Nuclear translocation of p52 started within the first 20 minutes and was maintained for 20  
30 hours. Accumulation of p100 in the nuclei of Ramos cells appeared within the first hour (Figure 2d, left panel), a process that was suggested to reflect induced

translocation of this protein in association with RelB to the nucleus (Yilmaz et al., 2003).

***Arrest of NIK synthesis in Ramos lymphoblastoid cells blocks CD27-induced activation of both the canonical and the alternative NF- $\kappa$ B pathways:***

5 To examine the role of NIK in activating various NF- $\kappa$ B forms through CD27, NIK synthesis in Ramos cells was arrested by infecting these cells with a lentiviral vector expressing a hairpin short interfering RNA (siRNA) capable of blocking NIK synthesis. Western analysis confirmed that both in transient and stable expression scenarios, the siRNA vector effectively arrested the synthesis of NIK (Figure 2f, top  
10 and middle panels). As expected from the reported participation of NIK in the NF- $\kappa$ B alternative pathway, treatment of the NIK<sup>(MINUS)</sup> Ramos cells with CD70 failed to induce translocation of p52 or of RelB to their nuclei. The translocation of p100 induced by CD70 in these cells was also significantly delayed (Figure 2d, right panel). NIK deficiency also resulted in inability of CD70 to induce I $\kappa$ B $\alpha$  degradation or  
15 nuclear p65 translocation (Figure 2e, right panel), both manifestations of the canonical pathway.

Control tests confirmed that the NIK<sup>(MINUS)</sup> Ramos cells express CD27 at levels comparable to those of normal Ramos cells and thus exhibit normal protein kinase C (PKC) activation upon CD27 triggering (Erlichman and Howard, 1999)  
20 (Figure 2g and its inset). These results indicated that the inability of NIK<sup>(MINUS)</sup> Ramos cells to activate NF- $\kappa$ B was not caused by aberrant CD27 function. To verify this observation, NIK expression was reinstated to the NIK<sup>(MINUS)</sup> cells (Figure 2f, bottom panel) by transfecting these cells with myc-tagged NIK cDNA to which conservative sequence changes were introduced to make it non-complementary with  
25 the NIK siRNA sequence. Although the I $\kappa$ B $\alpha$  and nuclear p52 levels in these cells were somewhat higher than normal (probably due to spontaneous NIK signaling as a result of its supra-normal expression level), the NIK<sup>(MINUS)</sup> reconstituted cells regained the ability to respond to CD70 with both an increase in nuclear p52 and a transient decrease in I $\kappa$ B $\alpha$  (Figure 2h), further confirming the pivotal role of NIK in  
30 activation of both the alternative and the canonical NF- $\kappa$ B pathways.

**EXAMPLE 3*****THE EFFECT OF NIK SUPPRESSION ON THE CANONICAL AND THE  
ALTERNATIVE NF- $\kappa$ B PATHWAYS***

The ability of CD40, BLyS, TNF, thapsigargin, or PMA to induce the  
5 canonical and the alternative NF- $\kappa$ B pathways was determined via western blot  
analysis which utilized antibodies directed to p100 p52, RelB, I $\kappa$ B $\alpha$  and p65. The  
effect of the presence of NIK in the cells was assessed by western blot analysis of the  
same molecules on NIK suppressed cells.

***Reagents:***

10 Production of hCD154 (CD40L) and hBLyS/BAFF was carried by large-scale  
transfection of human embryonic kidney (HEK) 293T cells with the relevant  
expression constructs (see below). In all tests, the conditioned media of the  
transfected cells were as described in Example 2. TNF, a gift from Dr. G. Adolf,  
Boehringer Institute, Vienna, Austria, was applied to cells at a concentration of 50  
15 ng/ml. Thapsigargin, 4 $\beta$ -phorbol-12-myristate-13-acetate (PMA), and  
phytohemagglutinin (PHA) were purchased from Sigma.

***Antibodies:***

The source of antibodies against p65, RelB, p52, p100, I $\kappa$ B $\alpha$  is described in  
Example 2 of the Examples section. Anti c-Rel was purchased from Santa Cruz  
20 Biotechnology. Anti- $\beta$  actin and anti-FLAG were purchased from Sigma. Anti-  
phospho-I $\kappa$ B $\alpha$  was purchased from Cell Signaling Technology.

***Cells:***

Ramos (Benjamin et al., 1982) cells were cultured as described in Example 2  
of the Examples section.

25 ***Expression vectors:***

The cDNAs for the extracellular domains of hCD154 (CD40L) (ATCC clone  
79814), and hBLyS/BAFF (Resgen clone 631119) were PCR-amplified from ESTs  
and cloned in fusion with a modified leucine zipper and FLAG tag (Fanslow et al.,  
1994) into pcDNA3 (Invitrogen). NIK suppression was generated as described in  
30 Example 2 of the Examples section.

*Transfections, immunoblotting and immunoprecipitations:*

Transfections, immunoblotting and immunoprecipitations were performed as described in Example 1 of the Examples section. Phosphorylated I $\kappa$ B $\alpha$  was detected after pretreatment (2 h) with the proteasomal inhibitor MG132 (25  $\mu$ M).

5        Ligand activation of normal and NIK<sup>(MINUS)</sup> Ramos cells was carried by stimulating cells for the time periods indicated: 0, 0.2, 0.5, 1 and 16 hours (Figure 3a), 0, 0.25, 1 and 16 (Figure 3a-b) with CD40L, for time periods of 0.3, 4 and 20 hours (Figure 3c-d) with BLYS, for time periods of 0, 0.3, 4 and 20 hours (Figure 3e) or 0, 0.3, 1, 4 and 20 hours (Figure 3f) with TNF. Cells stimulation by CD70 and TNF was  
10        carried for 0, 15 minutes and 4 hours (Figure 3g). Stimulation by Thapsigargin and PMA was carried for 0, 30 minutes and 4 hours (Figure 3h).

      Studies of the effects of various ligands of the TNF family on NF- $\kappa$ B activation in lymphocytes have demonstrated activation of both the canonical and the  
15        alternative NF- $\kappa$ B pathways by two ligands, the CD40 ligand (CD40L) (Berberich et al., 1994) (Coope et al., 2002) and BLYS/BAFF (Claudio et al., 2002) (Kayagaki et al., 2002) (Hatada et al., 2003). On the other hand, TNF, though capable of effectively triggering the canonical pathway, appears unable to trigger the alternative pathway (Matsushima et al., 2001) (Yin et al., 2001) (Dejardin et al., 2002) (Yilmaz et al.,  
20        2003). TNF, induces only a slight increase in nuclear p52, much less than that induced by ligands such as CD40L (Yilmaz et al., 2003) (Derudder et al., 2003), probably through stimulating the synthesis of p100 (de Wit et al., 1998). TNF also induces synthesis of RelB (Bren et al., 2001), which in part accumulates in the nucleus apparently through induced nuclear translocation of p100:RelB dimers (Yilmaz et al.,  
25        2003).

      The responses of the Ramos cells to CD40L, BLYS/BAFF, and TNF in the present study were consistent with those reports. All three ligands induced activation of the canonical pathway, as reflected in rapid nuclear translocation of p65 (Figures 3b, 3d, 3f, left panels). This translocation was associated with a decrease in I $\kappa$ B $\alpha$  (Figures 3b, 3f, left panels) or, in the case of BLYS/BAFF induction, phosphorylation of I $\kappa$ B $\alpha$  with no visible change in its cellular levels was detected (Figure 3d, left panel). CD40L and BLYS/BAFF also induced marked increase in nuclear p52 as well as in RelB, both reflecting activation of the alternative pathway (Figures 3a, 3c, left  
30

panels). TNF induced nuclear translocation of RelB, but only a slight increase in nuclear p52 (Figure 3e, left panel). Assessment of co-immunoprecipitation of various NF- $\kappa$ B proteins from nuclear extracts of Ramos cells confirmed that whereas CD70 enhances nuclear accumulation mainly of RelB:p52 but also of RelB:p100, TNF induces increased nuclear levels of RelB:p100 without increasing RelB:p52 (Figure 3g).

The induction of the NF- $\kappa$ B pathway by CD40L, BLyS/BAFF, TNF, thapsigargin and PMA was tested in Ramos cells in which NIK expression was arrested. All effects of CD40L and BLyS on NF- $\kappa$ B activation (nucleus translocation of p100, p52, RelB and p65 as well as I $\kappa$ B $\alpha$  degradation in the cytoplasm) were arrested in the NIK<sup>(MINUS)</sup> Ramos cells (Figures 3 a-d, right panels). In contrast, the induction of I $\kappa$ B $\alpha$  degradation by TNF and the resulting nuclear translocation of p65, as well as the induction of nuclear translocation of p100 and RelB, occurred in the NIK<sup>(MINUS)</sup> cells just as effectively as in the cells expressing NIK (Figures 3e, 3f, right panels). NIK depletion also had no effect on I $\kappa$ B $\alpha$  degradation in response to thapsigargin, an inhibitor of the sarco-endoplasmic reticulum Ca<sup>2+</sup>-adenosine triphosphatase that triggers activation of NF- $\kappa$ B through induction of endoplasmic reticulum stress (Pahl and Baeuerle, 1996), or to 4 $\beta$ -phorbol-12-myristate-13-acetate (PMA), an agent activating NF- $\kappa$ B through stimulation of PKC (Sen and Baltimore, 1986) (Figure 3h).

Besides the loss of unresponsiveness to the effects of CD70, CD40, and BLyS/BAff on NF- $\kappa$ B, the NIK<sup>(MINUS)</sup> Ramos cells also displayed some constitutive alterations of their basal NF- $\kappa$ B protein levels. They showed marked reduction of basal p52 and a significant decrease in RelB and c-Rel, as well as some reduction of p100 and I $\kappa$ B $\alpha$  (Figures 3e, 3f and 3i). Expression of all the above proteins depends in part on NF- $\kappa$ B activation (Hannink and Temin, 1990; Ten et al., 1992; Lombardi et al., 1995 and Bren et al., 2001). p65, whose expression is independent of NF- $\kappa$ B (Ueberle et al., 1993), occurred in the NIK<sup>(MINUS)</sup> cells in normal amounts (Figure 3i), although its basal nuclear level was reduced (Figure 2e). These constitutive alterations in the levels of NF- $\kappa$ B proteins in the NIK<sup>(MINUS)</sup> cells are reminiscent of those observed in lymphocytes of the *aly* mice (Yamada et al., 2000). They probably reflect

arrest of the effects of some autocrine mediator(s) that continuously activate NF- $\kappa$ B to a slight extent, in a NIK-dependent manner.

#### **EXAMPLE 4**

##### **5 THE EFFECT OF ANTIBODIES AGAINST THE PHOSPHORYLATED ACTIVATION-LOOP OF NIK ON I $\kappa$ B $\alpha$ DEGRADATION**

Antibodies against the phosphorylated activation-loop of NIK ( $\alpha$ p-NIK) were introduced into Ramos and BJAB cells creating instantaneously ablation of NIK activation. The effect of these antibodies on the induction of I $\kappa$ B $\alpha$  degradation by  
10 CD70 and CD40L and TNF was measured.

##### **Reagents:**

CD70 was produced as described in Example 2 of the Examples section. CD40L was produced as described in Example 3 of the Examples section. TNF was  
15 obtained as described in Example 3 of the Examples section. [ $\gamma^{32}$ P]ATP was purchased from Amersham Biosciences.

##### **Antibodies:**

The I $\kappa$ B $\alpha$  antibody is described in Example 2 of the Examples section. Anti-myc monoclonal antibody was purified as described in Example 1 of the Examples  
20 section. A monoclonal antibody against the phosphorylated NIK activation loop ( $\alpha$ -pNIK) was raised by immunizing mice with a KLH-coupled peptide corresponding to the NIK activation loop in which Thr559 was phosphorylated. The anti-NIK monoclonal antibody NIK-81 was raised as described in Example 2 of the Examples section. Both anti-NIK monoclonal antibodies were purified on affinity columns to  
25 which their corresponding peptides were coupled.

##### **Cells:**

The Ramos cells and HEK293T cells are described in Example 1 of the Examples section. BJAB cells (Clements et al., 1975) were cultured in RPMI medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100  $\mu$ g/ml  
30 streptomycin.

***Expression vectors:***

The cDNAs for the extracellular domains of CD70 was cloned as described in Example 2 of the Examples section. hCD154 (CD40L) and hBLyS/BAFF were cloned as described in Example 3 of the Examples section.

5      ***Transfections, immunoblotting and immunoprecipitations:***

Transfections, immunoblotting and immunoprecipitations were performed as described in Example 1 and Example 2 of the Examples section.

***Antibody transfection:***

10      Antibodies were transfected into cells in serum-free medium using the Pro-ject Protein Transfection Reagent Kit (Pierce), according to the manufacturer's instructions. Ligands were applied, in regular (serum-containing) medium (RPMI1640 WITH 10% FBS), 3–4 hours following antibody transfection.

***Assessment of uptake of FITC-tagged immunoglobulin:***

15      Assessment of FITC-tagged immunoglobulin uptake was carried via fluorescence microscopy 0, 1, 4 and 8 hours after transfection.

NIK activation was instantaneously ablated in order to exclude the possibility that the differences in ligand effects observed between the NIK<sup>(MINUS)</sup> and the normal  
20      cells are secondary to such constitutive alterations occurring in the cells as a consequence of prolonged NIK deficiency. NIK activation involves phosphorylation of its activation loop (Lin et al., 1998). A monoclonal antibody that was raised against a phospho-peptide corresponding to the phosphorylated NIK activation loop ( $\alpha$ -pNIK) and introduced into Ramos cells was shown to effectively block the *in-vitro*  
25      kinase function of NIK in a dose dependent manner (Figure 4a, top panel). Figure 4b illustrates that treatment of Ramos ~~cells~~ with a protein-transfection kit allowed effective, though transient, introduction of immunoglobulins into the cells. Introduction of the  $\alpha$ -pNIK antibodies into Ramos cells had no effect on the induction of I $\kappa$ B $\alpha$  degradation by TNF. However, the antibodies effectively blocked the  
30      induction of I $\kappa$ B $\alpha$  degradation by CD70 or CD40L (Figure 4c). In BJAB cells, CD40L induced I $\kappa$ B $\alpha$  degradation and this induction was significantly reduced when the  $\alpha$ -pNIK antibodies were introduced (Figure 4d). These findings further confirm that although NIK does not participate in activation of the canonical pathway by TNF, its

function in lymphocytes is crucial for activation of the canonical pathway by other ligands.

### **EXAMPLE 5**

#### **5 THE EFFECT OF CD70 ON ACTIVATION OF THE CANONICAL PATHWAY**

The abilities of TNF and CD70 to activate the IKK signalosome were compared. Their effect on the mechanisms of canonical pathway was examined in control and NIK deficient cells. The effect of the presence of NIK in the cells was assessed by western blot analysis of the same molecules on NIK suppressed cells.

#### **10 Reagents:**

CD70 is described in Example 2 of the Examples section, TNF is described in Example 3 of the Examples section.

#### **Antibodies:**

Anti-myc was purified as described in Example 1 of the Examples section.  
**15** Anti-I $\kappa$ B $\alpha$  and anti-NIK (NIK-81) are described in Example 2 of the Examples section. Anti-phospho-I $\kappa$ B $\alpha$  is described in Example 3 of the Examples section. Antibody against the phosphorylated NIK activation loop ( $\alpha$ -pNIK) is described in Example 4 of the Examples section. Anti-FLAG M2-beads were purchased from Sigma. IKK $\alpha$  (M280 & H744) from Santa Cruz Biotechnology, anti-IKK $\beta$  and anti-  
**20** IKK $\gamma$  from BD-Pharmingen.

#### **Cells:**

Ramos cells and PBMC cells are described in Example 2 of the Examples section.

#### **Expression vectors:**

**25** GST-I $\kappa$ B $\alpha$  was a gift from Signal pharmaceuticals. NIK suppressing vectors are described in Example 2 of the Examples section.

#### **Transfections, immunoblotting and immunoprecipitations:**

Transfections, immunoblotting and immunoprecipitations were performed as described in Example 2 and Example 3 of the Examples section. For  
**30** immunoprecipitation of the CD70/CD27 ligand-receptor complexes from resting PBMC and Ramos cells, cell lysates were prepared as described for the kinase tests aforementioned and incubated for 4 hours at 4 °C with 25  $\mu$ l of 50% M2-FLAG



agārose beads per ml of lysate. The TNF–receptor complex was precipitated as described (Zhang et al., 2000). The signalosome from cell lysates was immunoprecipitated using a 1:1 mixture of two different antibodies against IKK1 (M-280 and H-744, Santa Cruz). Immunoprecipitation was allowed to continue for 2 hours at 4 °C, using 10 µg of anti-IKKα antibodies adsorbed to 25 µl of 50% protein-A–Sepharose beads per ml of lysate.

*In vitro* GST-IκBα phosphorylation in normal and NIK<sup>(MINUS)</sup> Ramos cells was carried by stimulating cells for the time periods indicated (0, 0.25 and 4 hours) (Figure 5a) with CD70 or TNF.

#### Kinase tests:

The *in-vitro* IKK kinase activity of the receptor complexes and cytoplasmic signalosome complex, using a bacterially expressed GST-IκBα (1–54) (Uhlik et al., 1998 and Dejardin et al., 2002)) as substrate, was assessed as previously described (Uhlik et al., 1998 and Dejardin et al., 2002). Briefly, 2–4 × 10<sup>8</sup> Ramos cells were lysed by rotation for 30 minutes at 4 °C in lysis buffer (20 mM HEPES pH 7.6, 250 mM NaCl, 0.5% NP-40, 20 mM β-glycerophosphate, 1 mM EDTA, 20 mM p-nitrophenyl phosphate, 0.1 mM sodium vanadate, 2 mM sodium fluoride, 1 mM DTT, 1 mM PMSF, and 1× complete protease inhibitor cocktail). Cellular debris was removed by centrifugation at 10,000 × g and the lysate was pre-cleared with protein A/G beads on to which rabbit/mouse pre-immune serum was adsorbed, and then subjected to immunoprecipitation for 2 hours at 4 °C. The immunoprecipitates were washed four times with lysis buffer and twice with kinase buffer (20 mM HEPES pH 7.6, 20 mM MgCl<sub>2</sub>, 20 mM β-glycerophosphate, 1 mM EDTA, 2 mM p-nitrophenyl phosphate and 2 mM DTT). The kinase reaction was allowed to proceed by incubating the immunoprecipitated proteins bound to 20-µl beads in kinase buffer (40 µl) containing 1 µg GST-IκBα (1–54) and 5 µci [γ<sup>32</sup>P]ATP at 30 °C for 30 minutes. The kinase activity of NIK was assessed under the same conditions, using myc-tagged NIK that had been over-expressed in transfected HEK293T cells and immunoprecipitated using anti-myc antibody. The kinase test was carried out in the presence of α-pNIK or IgG as control, following pre-incubation of the immunoprecipitate with the antibodies for 1 hour at 4 °C. Samples of the kinase reactions were separated on SDS–PAGE, transferred to nitrocellulose membranes,

visualized by autoradiography and the indicated proteins were subjected to western blot analysis.

*Activation of the canonical pathway by CD70 is associated with a selective  
5 NIK-dependent recruitment of IKK1 to CD27:*

The critical event in the canonical pathway is stimulation of the I $\kappa$ B-kinase activity of the IKK signalosome complex. The abilities of TNF and CD70 to activate the IKK signalosome were compared in order to examine the differences in the mechanisms of canonical pathway activation by these two ligands.

10 Both TNF and CD70 were found to enhance the *in vitro* kinase function of the IKK signalosome, manifested in phosphorylation of GST-I $\kappa$ B $\alpha$  (Figure 5a), as well as in self-phosphorylation of the IKKs and phosphorylation of NEMO (Figure 5b). However, whereas activation of the signalosome by TNF was not affected by NIK deficiency, the effect of CD70 on the signalosome was aborted in the NIK<sup>(MINUS)</sup> cells  
15 (Figure 5a, b).

Following activation of the signalosome by TNF all three components of the signalosome (IKK1, IKK2, and NEMO) are recruited, in about the same ratio as that found in the complex that they form in the cytosol. Signalosome recruitment to the p55 TNF receptor upon TNF treatment is induced just as effectively in NIK<sup>(MINUS)</sup>  
20 cells as in the wild-type cells (Figure 5c, right panels). CD70 appears to induce the recruitment of only one of the three components of the canonical signalosome, IKK1, to CD27 and its recruitment to CD27 upon CD70 treatment is completely abolished in the NIK<sup>(MINUS)</sup> cells, indicating that NIK function is required for this process (Figure 5c, left panels). Similar selective recruitment of IKK1 to CD27 upon CD70  
25 treatment was also observed in PBMC (Figure 5d). In the case of both the p55 TNF receptor and CD27, the kinase activity of the receptor-associated IKKs was weaker than that of the cytoplasmic signalosome (phosphorylation of GST-I $\kappa$ B $\alpha$  in top and bottom panels of Figure 5c and also in right and left panels of Figure 5d), suggesting that the recruitment does not result in full activation of the signalosome but merely in  
30 initiation of the activation process.

The earliest known event in activation of the signalosome by TNF is its recruitment to the p55 TNF receptor, a process facilitated by recruitment of the adapter protein RIP

and TRAF2 to that receptor. CD70 does not induce recruitment of RIP to CD27. however, as shown in figure 6a it dose induce recruitment of TRAF2. Interestingly, the TRAF2 molecules recruited to CD27 displayed extensive electrophoretic pattern modification, probably corresponding to ubiquitination. CD70 also induced  
5 recruitment of the signalosome. Recruitment of the signalosome to the TNF receptor was prolonged, whereas association of the three components of the signalosome with CD27 could be observed only for a few minutes. At later time points the amounts of IKK2 and NEMO in the CD27 complex sharply decreased. Surprisingly, however, the amounts of IKK1 associated with the receptor remained high for a long time (Figure  
10 6a, left panel). Similar selective maintenance of IKK1 in association with CD27 after CD70 treatment was also observed in PBMC (Figure 6b).

Both TNF and CD70 also induced recruitment of all three components of the canonical NF- $\kappa$ B complex I $\kappa$ B $\alpha$ , p65 and p50. Although p100 processing is induced  
15 by CD70 and not by TNF, recruitment of p100 to its receptor was induced by TNF but not by CD70 (Figure 6a, right panel). This recruitment was previously suggested to occur through binding of the death domain in p100 to that in the p55 TNF receptor-associated adapter protein TRADD, and appears to serve not to activate NF- $\kappa$ B, but to amplify death induction by this receptor trough caspase-8 activation (Wang et al.  
20 2002).

In NIK- cells, recruitment to the p55 TNF receptor occurred just as effectively as in the wild type cells (Figure 6c, right panel). In contrast, recruitment of the signalosome components to CD27 was completely abolished (Figures 6c, d, left panels). Introduction of the wild type NIK, but not of "kinase-dead" NIK, to the NIK- cells  
25 reintated the recruitment in response to CD70 (figure 6d, middle and right panels).

CD70, but not TNF, also induced recruitment of NIK to its receptor. This recruitment could be observed both in cells expressing the wild type enzyme and in those that expressed its "nik-dead" mutant (figure 6d). Thus, while the recruitment of the signalosome components to the CD27 depends on NIK kinase function, recruitment of  
30 NIK itself to the receptor seems to occur independently of its enzymatic activity.

**EXAMPLE 6*****SPECULATIVE MODEL OF THE MECHANISMS INITIATING NF- $\kappa$ B  
ACTIVATION BY TNF AND CD70***

5       After comparing the initiating events in the NIK-independent activation of NF- $\kappa$ B by TNF and in the NIK-dependent activation by CD70 we show that the participation of NIK in activation of the canonical pathway is restricted to the effect of specific inducers.

10       Activation of NF- $\kappa$ B by the p55 TNF receptor is associated with recruitment all of the signalosome to it, and in the process the signalosome components interact with TRAF2 and RIP (Zhang et al., 2000) (Devin et al., 2000) (Devin et al., 2001). Activation of the canonical pathway by CD70, like its activation by TNF, is associated with the recruitment of the signalosome component IKK1, IKK2, and NEMO to the receptor complex. Unlike TNF, however, CD70 also induces  
15       recruitment of NIK and the signalosome components, IKK2 and NEMO to its receptor.

Recruitment of the signalosome along with NIK to CD27 is followed shortly by a sharp decrease in both IKK2 and NEMO in the receptor complex. Both IKK1 and NIK, however, remain associated with the receptor for a long time. The latter form of  
20       CD27 complex probably serves to initiate the alternative pathway (See hypothetical model in Figure 7).

Like the recruitment of the whole signalosome to CD27, the subsequent preferential association of IKK1 with the receptor cannot be observed in cells devoid of NIK or in cells expressing non-functional NIK mutants.

25       Apparently, these two stages in the recruitment by CD70 are mechanistically linked, thereby ensuring that initiation of the NIK-dependent alternative activation pathway is coupled to that of the canonical pathway.

In accordance with the invention, NIK was found to bind SIVA, a protein that  
30       appears to associate with CD27 (Prasad et al., 1997). Prior art studies have shown that SIVA mediates the induction of cell death by CD27. However, death induction seems to be restricted to only one of the two known SIVA splice variants, namely SIVA1, which contains a death-domain motif (Yoon et al., 1999). We show that both SIVA2

(which lacks a death domain) and SIVA1 bind to NIK through a C-terminal region common to the two splice variants and potentially enhance the activation of NF- $\kappa$ B by over-expressed NIK. The fact that SIVA does not enhance the activation of NF- $\kappa$ B by the non-functional NIK *aly* mutant gives credence to the possibility that SIVA plays some role in NF- $\kappa$ B activation by NIK. Whether this role indeed concerns the triggering of NIK function by CD27, or some other aspect of NIK function, remains to be clarified. None of the other receptors so far shown to involve NIK in their signaling is known to bind SIVA. These receptors might involve some other adapter proteins in NIK activation.

The activities mediated by the canonical and the alternative NF- $\kappa$ B activation pathways, though distinct, are also interrelated. The NF- $\kappa$ B dimers generated by the two pathways recognize different DNA sequence motifs, and thus, by affecting different promoters, can control the expression of different genes (Perkins et al., 1992) (Lin et al., 1995) (Dejardin et al., 2002) (Hoffmann et al., 2003). The two pathways also possess different kinetic features. Activation of the canonical pathway is rapid and it turns out to be transient mainly because it induces the synthesis of NF- $\kappa$ B-inhibitory proteins such as I $\kappa$ B and NF- $\kappa$ B2/p100. In contrast, the alternative pathway reaches effective activation only several hours following stimulation and remains active for a long time. These differences allow the two pathways to control different sets of genes, which serve different functions. Thus, consistently with their rapid induction, the dimers activated by the canonical pathway control a set of genes that mediate the early innate immune response, whereas the dimers generated by the alternative pathway control activities that contribute in a variety of ways to the prolonged and more slowly induced adaptive immune response. These differences in function correlate with the functions ascribed to the ligands that control the two activities. Pro-inflammatory cytokines such as TNF and interferon IL-1 can potentially stimulate the canonical pathway, yet have little ability, if any, to stimulate the alternative one, whereas ligands such as LT $\alpha$ 1 $\beta$ 2, CD40L, BLyS/BAFF, and CD70, which control adaptive immunity, can, in addition to activating the canonical pathway, also effectively trigger the alternative one.

However, the mere fact that the same ligands that activate the alternative pathway also activate the canonical one allows for functional interactions between the

genes regulated by these two pathways. Moreover, the two signaling pathways interact, affecting each other's activation. Induction of the canonical pathway triggers the synthesis of p100 as well as of RelB (de Wit et al., 1998) (Bren et al., 2001), which together form the precursor dimer affected by the alternative pathway, and thus  
5 potentiate the latter. Conversely, since in addition to binding to RelB p100 also associates with the dimers controlled by the canonical pathway (p65:p50 and c-Rel:p50) and thus blocks their function, its processing by the alternative pathway helps to perpetuate the activation of the canonical pathway.

To allow for coordination of the activation mechanisms for the two  
10 functionally distinct yet interacting sets of NF- $\kappa$ B dimers by the same inducer, they need to be controlled by both common and distinct regulatory elements. Other studies have disclosed several components unique to the alternative or to the canonical pathway. We show, for the first time, that NIK can serve as a common participant in these two distinct NF- $\kappa$ B-activation pathways.

15 Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims. All publications, patents and patent applications and  
20 GenBank Accession numbers mentioned in this specification are herein incorporated in their entirety by reference into the specification, to the same extent as if each individual publication, patent or patent application or GenBank Accession number was specifically and individually indicated to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be  
25 construed as an admission that such reference is available as prior art to the present invention.

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